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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

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VOLUME XXXIV 1940

PART II, pp. 781 to end

CAMBRIDGE

AT THE UNIVERSITY PRESS

1940

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OBITUARY NOTICE

PATRICK PLAYFAIR LAIDLAW

(1881–1940)

Sir Patrick Laidlaw died in his sleep on 19 March 1940 in his 59th year. He was educated at the Leys School and St John's College, Cambridge, and at Guy's Hospital. After a short period as Demonstrator in Physiology at Guy's, he joined Dr (now Sir) H. H. Dale, in 1909, at the Wellcome Physiological Research Laboratories. In 1914 Laidlaw was appointed Professor of Pathology at Guy's Hospital, but in 1922 his association with Dale was resumed at Hampstead and, at the time of his death, he was head of the Department of Experimental Pathology, and Deputy Director, at the National Institute for Medical Research.

Laidlaw was elected a Fellow of the Royal Society in 1927 and awarded a Royal Medal in 1933. He was elected a Fellow of the Royal College of Physicians in 1934 and knighted in 1935. He was Rede Lecturer at Cambridge in 1938 and, a few days before his death, he was elected to an honorary fellowship at his old College, St John's, Cambridge.

Laidlaw's international reputation as an investigator of the highest class is assured, and all who knew him recognize that the high regard in which all his work is held does not rest upon his achievements in a single or restricted field of research. While it is the case that physiologists will associate his name with his work on amines, much of it carried out in association with Dale, and pathologists with his pioneer and fundamental researches on virus diseases, and that his enduring fame will be associated with his contributions to knowledge in these important fields (which are dealt with in detail elsewhere), yet, throughout a busy and varied life, Laidlaw found time for research in many other subjects. His interest in biochemistry began at Cambridge where he was a member of one of the earlier of Prof. (now Sir) F. G. Hopkins's classes, and that interest was maintained throughout his life. Quite a large part of the limited floor space in his laboratory at Hampstead was occupied by a chemical bench, and the ordinary biochemical procedures were as familiar to Laidlaw as those of physiology, pathology and bacteriology. He was an original member of the Biochemical Society and four of the twelve papers on biochemical subjects which he published appear in the Society's Journal.

Perhaps the most outstanding, and certainly the most arresting, of his biochemical papers was his first, on blood pigments, which contained the results of investigations carried out while he was still an undergraduate. In this paper he showed that the stability of the iron in the blood pigment molecule is dependent on the presence or absence of oxygen, offered an explanation of the formation of haematoporphyrin in cases of haematoporphyrinuria, prepared haematin from haematoporphyrin and proved its identity with the natural product, prepared turacin by boiling haematoporphyrin with ammoniacal copper solution and showed its identity with the natural product. This short paper of eight pages is remarkable, not only for the importance of the results obtained, but as revealing all those qualities as an investigator which characterized Laidlaw's subsequent work.

Another important investigation dealt with the effect of asymmetric nitrogen atoms on physiological activity. Cushny and others had shown that optical

isomers, due to asymmetric carbon atoms, have very different physiological activities. Laidlaw was the first to show that an asymmetric nitrogen atom may also exercise a profound effect on physiological activity. He studied the α and β varieties of *l*- and *d*-canadine methochlorides and showed that in the former case the β variety was twelve times, and in the latter case three times, as active as the α form.

Laidlaw's other biochemical investigations covered a varied field. He carried out a very detailed study of the possible relationship between the chemical structure of certain *iso*quinoline derivatives and their physiological action and suggested a law of relationship for one series. He also studied the physiological action of 3-β-aminoethylindole and demonstrated the identity of the synthetic product with that prepared from tryptophan by bacterial action, and with A. J. Ewins he investigated the fate of this and other amines in the animal organism. With the late H. W. Dudley he investigated the antigenic structure of the tubercle bacillus, with W. W. Payne he described a method for the determination of small amounts of calcium, and with H. H. Dale a method for the preparation of secretin; and it is of interest to note that some of the procedures used for the preparation of secretin have since been applied to the purification and attempted separation of other physiologically active substances.

Laidlaw had many traits of character which endeared him to all who were privileged to know him. Most attractive of all, perhaps, was his shyness which remained, to some extent, even with his most intimate friends. He was, however, the most approachable of men, and there can be few who have been consulted so often and on such a variety of subjects. Among his colleagues at the National Institute, whatever their special interest, it was almost a routine to "ask P. P." if help or advice were needed; and this service was not restricted to his immediate colleagues. His help, advice and criticism were available for all who sought it, and, although for him travelling was not easy, his visitors were many and they came from far and near. His vast knowledge in so many fields of scientific activity, his wide experience of experimental technique and design, his sound judgment and keenly critical mind, and his kindliness, modest diffidence and approachability will remain a lasting memory to Laidlaw's colleagues and friends.

P. H.

94. ESTIMATION OF LIGNIN IN TANNIN MATERIALS

By JAGESHWAR GOPAL SHRIKHANDE

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(*Received 30 January 1940*)

The necessity for an investigation of this problem arose when the question of losses of lignin during decomposition of green materials was under examination. Lignin percentages obtained by the 72 % H_2SO_4 method after a pretreatment by 5 % H_2SO_4 looked strikingly high and the results became more suspicious when they were considered along with the decomposition undergone by the tannin materials under investigation. For instance, for materials of lignin content of 39 % to suffer a loss of about 35 % in dry matter during a period of 35 days appeared anomalous because it is known that highly lignified tissues are very slow to decompose. Secondly, such a lignin content in green leafy materials like tea leaf prunings or refuse tea looks absurd when compared with even the most highly lignified tissues like coconut coir or certain woods which only rarely attain such a high degree of lignification. It thus became apparent that 5 % H_2SO_4 hydrolysis as a pretreatment for materials containing tannin is undesirable since instead of removing any extraneous material which interferes with lignin estimation it probably renders it insoluble.

The various substances known to interfere with the determination of lignin in plant materials in general are the pentose groups of the hydrolysable polysaccharides, proteins, substances of an aldehydic or ketonic character and fatty and resinous substances. Waxes and fats are removed by extraction with a mixture of benzene and alcohol. Since no serious objection to the use of acid pretreatment has been raised this has been widely employed for a long time to minimize pentose and protein disturbance [Norman & Jenkins, 1934; and Norman, 1937]. Although acid hydrolysis as a pretreatment for a wide range of materials has been found handy, it can by no means be universally adopted. Exceptions have occasionally been recorded. Bamford & Campbell [1936] obtained an increase in the apparent lignin from wood of beech and African pencil cedar after a treatment with 5 % sulphuric acid. Cohen [1936] found it difficult to estimate the lignin in eucalyptus wood correctly by the common procedure. Ritter & Barbour [1935] observed an interference due to the presence of tannins of the catechol group in lignin determination of certain woods. The question arises whether other tannins, or even quite different substances present in different materials are rendered insoluble in the presence of dilute acid. The existence of such special minor substances affecting the lignin determination calls for further pretreatment. After surveying the literature it was noted that no other tannin materials except those of the catechol group have been studied. It was therefore thought desirable to make comparisons between catechol tannins and those of other groups.

Classification of tannin materials

As a preliminary to the investigation it was necessary to classify the various tannin materials into certain definite groups. This should give a clue to whether all forms of tannin behave alike in lignin estimations or whether the catechol group is specific with regard to such a hindrance. The present classification is based on the suggestion of Nierenstein [1934] and Haas & Hill [1921] and chemical tests described by Proctor [1903]:

(1) Condensed tannins → Catechol tannins → Tea leaf and refuse tea.
→ Pyrocatechol tannin → *Pterocarpus indicus.*

(2) Hydrolysable tannins → Ellagi-tannins → *Terminalias* and *Caesalpinias.*

(3) Substituted pyrogallol derivatives → Pyrogallol tannin → *Gordonia imbricata.*

It will thus be seen that the materials under investigation represent a major portion of the various types of tannins given by Nierenstein [1934]. Such a classification should enable one to study the method more closely and to develop a pretreatment which might embrace all groups of tannins.

Experimental

(*a*) *Application of the common method*

Results in Table 1 (*a*) leave no doubt that the pretreatment with 5% H_2SO_4 is faulty and is inapplicable, with one or two exceptions, to such types of materials on account of the high percentages of apparent lignin. Water extraction of the hydrolysed product from a few of the materials was also tried with a view to dissolving out any extraneous matter unaffected. Water does seem to lower the values for apparent lignin, perhaps by extracting some of the substances unattacked by acid. But they still appear to be appreciably high. It is probable that tannin, being a phenolic body, is precipitated in the presence of H_2SO_4 and is thus rendered insoluble in water. It is then ultimately estimated as lignin by the 72% H_2SO_4 method.

Table 1. *Apparent lignin in materials after various extractions as pretreatments for lignin estimation*

Results expressed on 100 g. original material on ash free basis

1	2	3	4	5	6	7	8	9
	(*a*)		(*b*)		(*c*)			
Material	H_2SO_4	H_2SO_4-H_2O	H_2O	H_2O-H_2SO_4	95% alcohol	Alcohol-H_2SO_4	Alcohol-H_2O	Alcohol-H_2O-H_2SO_4
Tea leaf	39·84	31·08	34·12	25·40	34·22	29·80	28·31	23·93
Refuse tea	38·70	29·04	32·45	28·44	41·90	29·70	31·47	26·61
Pterocarpus indicus	21·63	—	25·68	21·35	—	—	—	16·94
Caesalpinia Bonduc	30·39	28·97	30·32	27·56	29·07	25·87	26·73	23·85
C. coriaria	25·71	21·81	24·43	20·59	21·64	18·67	17·84	15·50
C. sappan	26·11	—	27·93	25·18	—	—	—	17·70
Terminalia Belerica Roxb.	27·99	—	30·37	27·87	—	—	—	23·70
T. Chebula	31·93	—	—	—	—	—	—	—
Gordonia (probably *imbricata*)	18·92	—	18·98	17·19	—	—	—	12·42

(b) Proposed modification of the common method

The modification is based on the fact that the tannins in these materials are extractable by hot water. In fact tannins in plant materials are estimated in the hot water infusion obtained under certain specified conditions [Shaw, 1935]. There is therefore every reason to suppose that a preliminary treatment with hot water ought to remove any tannins if they are the other interfering substances. The hemicelluloses and proteins can then be hydrolysed in the water-extracted residue by dilute H_2SO_4 thus removing almost all the suspected disturbing factors in lignin determination of tannin materials. Working on such a basis, all the materials were first extracted with hot water. Lignins were first determined on this residue. It will be noted from Table 1 (*b*) that in general the apparent lignin in the water-extracted residue is practically of the same order as after acid treatment (Table 1 (*a*)) with the exception of tea leaf and refuse tea, materials which are classified as catechol tannins. Lignins for these two are lower on water-extracted residue than on the hydrolysed product. Thus catechol tannins react in a different way from the other types in that they appear to be insoluble in the presence of dilute acid. Lower apparent lignins in the residue after water extraction of catechol tannins are undoubtedly due to removal of tannin and other water-soluble extraneous substances; but with other materials, on account of the similarity between acid and water treatments, it looks as though the disturbances caused by hemicelluloses and proteins which are minimized by acid hydrolysis mask the lowering in apparent lignin due to tannin water extraction. But such a masking of factors is undesirable in any accurate method of analysis. Therefore, after removing tannins it is equally essential to remove proteins and hemicelluloses which are hardly extractable by water. Evidence of the water-insoluble nature of proteins, at any rate, in tea leaf and refuse tea has been given by Shaw [1934]. Similar results have also been obtained in these laboratories. Dilute H_2SO_4 hydrolysis is therefore adopted for water-extracted residues to get rid of any hemicellulose and protein interference. Some idea of the abundance of proteins in these materials can be obtained from the nitrogen figures quoted in Table 3. Apparent lignins in the hydrolysed products are given in column 5 of Table 1 (*b*). These values look more reasonable and are perhaps closer to the true lignins in these materials, not only because they are lower, but because they conform to the idea previously mentioned that easily decomposable materials are not high in lignin content. The proposed method, therefore, consists in extracting the tannin material with boiling water for 1 hr. and subsequently hydrolysing with 5% H_2SO_4 for a similar period to get rid of tannin, protein and pentose interference, before proceeding with the 72% H_2SO_4 method of lignin estimation originally described by Ost & Wilkening [1910].

(c) 95% alcoholic extraction as a preliminary to the proposed method

While the above method was under investigation the paper by Ritter & Barbour [1935] became available and it was then considered expedient to try their method of alcohol and water extractions in relation to tannin interference. Accordingly all the materials were extracted with 95% alcohol for 4 hr. in a Soxhlet apparatus as recommended by them. Instead of extracting the residue after alcohol extraction with water alone, it was thought desirable simultaneously to study the effect of hydrolysis with dilute acid, and also the effect of combining both the water and acid extractions on the alcohol-extracted residues. Apparent lignins were then determined by the usual acid method in the four different residues. The results are incorporated in Table 1 (*c*). The

results with alcohol extraction alone are apparently too high because although alcohol extracts the tannin it has practically no effect on proteins and hemicelluloses. Subsequently water has almost the same effect as dilute H_2SO_4. This may be explained by considering the fact that any tannin which would ordinarily have been affected by dilute acid has already been extracted by alcohol and hence does not interfere with lignin. Such similarity has been commented upon by Norman [1937, 1]. Judging from the trend of these results it seems that neither of the two individual extractions with water or H_2SO_4 following upon alcoholic extraction is so effective as the combined action of both because the apparent lignins are still appreciably higher than those in column 5 of Table 1 (*b*). It seems therefore that water and H_2SO_4 treatments following successively upon alcoholic extraction are necessary for a satisfactory working of the proposed method. When alcohol-sulphuric acid figures are compared with water-sulphuric acid in Table 1 it looks as though water is superior to alcohol in its extracting capacity for catechol tannins. Water on this ground would have been sufficient to get over the tannin interference but for the disturbance caused by chlorophyll which is reported later in the paper.

In view of these results the lignin determinations on the remaining species were confined to the residue after the combined alcohol-water-sulphuric acid pretreatment. When such values are compared with those in column 5, Table 1 (*b*) it will be noted that they are lower in general by a mean of about 4 % than those with water-sulphuric acid treatments. Obviously alcohol has extracted something extraneous which was unaffected by the solvent action of water and sulphuric acid and which acted as a disturbing factor in addition to tannins, proteins and hemicelluloses in the lignin determination. Such lower values are possible under two circumstances—(1) alcohol dissolves some of the "soluble lignin", (2) chlorophyll and other pigments are removed by 95 % alcohol. The dissolution of soluble lignin during alcoholic extraction is possible on the evidence given by Mehta [1925] who worked with certain woods. But in the present investigation it is felt that chlorophyll, being ketonic in nature, is the apparent cause for an additional interference which is removed by alcohol. Evidence to this effect has been obtained and is contained in the following section.

(*d*) *Some constituents in tannin materials which cause interference during lignin estimations*

Table 2 records data for the possible interfering constituents either reacting independently or in association with others during lignin determinations by the 72 % H_2SO_4 method. Commercial tannic acid was the first to be tested because that is the most easily procurable tannin in pure condition. Caffeine, which is largely present in tea leaf and refuse tea, was another which is also easily obtainable. Neither of them independently or conjointly produced any precipitate in presence of 72 % H_2SO_4. But this evidence does not preclude the possibility of tannic acid as a phenolic body or caffeine as a ketonic body condensing with lignin in the presence of strong acid. In fact Norman [1937, 2] obtained substantial increases in the apparent lignin of wheat straw in presence of tannic acid. Since it is known that the tea tannin is different in composition from ordinary tannic acid, it was decided to isolate it in pure condition by Shaw's method [1935] for the present investigation. It will be noted that this tannin produced a precipitate with H_2SO_4 and four times as much with caffeine. The question of chlorophyll interference which was raised in the last section was

tested on two chlorophyll preparations reacting either alone with caffeine or with caffeine and theotannin. One sample was prepared in the laboratory by Willstätter's method and the other one was a B.D.H. solution of water-soluble chlorophyll. Both the samples threw down a precipitate in strong H_2SO_4. The effects of caffeine and theotannin were the same on chlorophyll although caffeine alone gave no precipitate with H_2SO_4. When caffeine and theotannin were both present the precipitate with acid was still greater. It is not difficult to explain chlorophyll interference from these results because chlorophyll contains a ketonic group and substances with ketonic or aldehydic groups are known to condense readily with lignin in presence of strong acids. Some condensation products might also have been formed between theotannin and caffeine, the former being phenolic like lignin and the latter a ketonic substance. The resultant product might fall into a group of tannins described by Nierenstein [1934] as caffe-tannins and might consequently be estimated as lignin. Wherever theotannin and caffeine were present together the filtrate after lignin filtration became turbid on standing and started throwing down a precipitate. This precipitation of the filtrate might be attributed to the oxidation products of theotannin which are being slowly brought down. These observations clearly indicate the causes why tea leaf and refuse tea in particular give disproportionately high and erroneous values for apparent lignins if improperly treated. Such a formidable grouping of ketonic, phenolic and quinone bodies in the form of caffeine, chlorophyll and theotannin is rarely met with in other plant tissues, or even in the other tannin materials.

Table 2. *Apparent lignin from different constituents in tannin materials after 72% sulphuric acid treatment*

Material	Apparent lignin (actually obtained) g.	Remarks
0·2 g. Tannic acid	Nil	Does not dissolve in 72% H_2SO_4 but goes into solution on dilution
0·2 g. Caffeine	Nil	Dissolves in 72% H_2SO_4
0·2 g. Tannic acid + 0·1 g. caffeine	Nil	Same as tannic acid
0·2 g. Theotannin	0·0021	Dissolves in 72% H_2SO_4 with deep yellow colour. Precipitate forms overnight colour changing to reddish yellow. During heating on dilution colour changes to amber
0·2 g. Theotannin + 0·1 g. caffeine	0·0079	Same as theotannin
0·11 g. Chlorophyll (lab. preparation)	0·0610	Gives a clear green solution with 72% H_2SO_4. Precipitate forms overnight
0·5 ml. Water-soluble B.D.H. chlorophyll	0·0050	Precipitates at once in 72% sulphuric acid
0·5 ml. Water-soluble B.D.H. chlorophyll + 0·1 g. caffeine	0·0151	Same as B.D.H. chlorophyll
0·5 ml. Water-soluble B.D.H. chlorophyll + 0·1 g. theotannin	0·0152	Forms precipitate with 72% H_2SO_4 with amber colour
0·5 ml. Water-soluble B.D.H. chlorophyll + 0·1 g. caffeine + 0·1 g. theotannin	0·0235	Same as B.D.H. chlorophyll and theotannin

(*e*) *Final procedure for estimation of lignins in tannin materials*

2 g. of the oven-dry material passing through a 80-mesh sieve are extracted in a Soxhlet apparatus with 200 ml. 95% alcohol for 4 hr. The extracted residue is transferred to a round-bottomed flask of 300 ml. capacity and extracted with

150 ml. boiling water under a reflux for 1 hr. The contents of the flask are then filtered and the residue is transferred back to the flask and hydrolysed with 150 ml. 5% H_2SO_4 for 1 hr. The hydrolysed product is then collected in a weighed sintered glass crucible after being washed free of acid. The residue is weighed and well powdered. 0·2 g. lots are weighed out into 1000 ml. beakers and treated with 20 ml. 72% H_2SO_4. The powder is well mixed with the acid and allowed to stand overnight, at a temperature varying between 20 and 22° (this is the range between 4 p.m. and 8 a.m. the following morning). The following morning the contents of the beakers are made up to 800 ml. with water and boiled for 2 hr., the volume being maintained by occasional addition of water. It is best to leave the beakers overnight so that the lignins settle down, thereby facilitating filtration. Solutions are invariably cloudy and are difficult to filter. This may be attributed to large amounts of protein in these materials. Such a difficulty was also noted by Norman & Jenkins [1934] with large amounts of protein at the time of studying protein interference in lignin determination. The filtration is done under suction in Gooch crucibles with No. 42 Whatman filter paper. After drying, the precipitates are weighed and ignited and the lignins calculated on an ash-free basis.

(f) Relationship between lignin, nitrogen and tannin in tannin materials

When lignins and nitrogen contents of the different materials are arranged in descending order as in Table 3, the rank in respect of nitrogen and lignin coincides in 5 of the 8 examples. This is not a sufficient coincidence to establish a valid correlation but further exploration of a larger number of samples might clarify a relationship between the two if such exists. No relation is noticeable between tannin and lignin. This was unexpected because some evidence exists of relationship between lignin and phenolic residues of tannin, Norman [1933].

Table 3. *Relationship between lignin, nitrogen and tannin in tannin materials*

Material	Lignin % alcohol-H_2O-H_2SO_4	Lignin % H_2O-H_2SO_4	Nitrogen %	Tannin %
Refuse tea	26·61	28·44	3·97	18·30
Pterocarpus indicus	16·94	21·35	3·50	3·47
Caesalpinia Bonduc	23·85	27·56	3·21	4·71
Tea leaf	23·93	25·40	2·97	7·93
Caesalpinia sappan	17·70	25·18	2·73	1·65
Terminalia Belerica	23·70	27·87	2·41	14·03
Caesalpinia coriaria	15·50	20·59	1·99	18·23
Gordonia imbricata	12·42	17·19	0·78	15·31

SUMMARY

1. A number of tannin materials were examined with regard to the question of tannin interference in lignin determinations. They were classified in three major groups so as to obtain information as to whether such interference is specific to any particular group. The results indicate that tannin interference is more a question of degree than of kind although catechol tannins cause the greatest disturbance.

2. 5% H_2SO_4 pretreatment instead of removing the extraneous material actually enhances the apparent lignin.

3. 1 hr. boiling with water to remove the tannin and subsequent hydrolysis with 5% H_2SO_4 to get over pentosan and protein interference bring down the

apparent lignins within reasonable limits. Since 95 % alcohol extraction revealed one new cause of interference in the form of chlorophyll, its value as a preliminary extracting agent is indicated.

4. Vegetable tannins, caffeine and chlorophyll should be added to the list of substances which are known to interfere in lignin determination of green materials.

5. The exceptionally high values of apparent lignins for tea leaf and refuse tea with 5 % H_2SO_4 as the only pretreatment are due to a formidable grouping of phenolic and ketonic groups in the form of theotannin, caffeine and chlorophyll. Such grouping is rarely met with in other plant tissues including other tannin materials.

6. Successive extractions of any tannin material with 95 % alcohol, hot water and 5 % H_2SO_4 make the Ost & Wilkening [1910] method more adaptable to such materials. This procedure is desirable irrespective of the nature of the tannin.

7. No relationship is discernible between tannin content and lignin. A superficial connexion between nitrogen and lignin requires further and more extensive work in order to clarify the position.

I am thankful to Dr R. V. Norris, the Director, for permission to publish this paper and to Dr T. Eden, Agricultural Chemist, for criticizing the manuscript.

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95. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

66. PENICILLIOPSIN, THE COLOURING MATTER OF *PENICILLIOPSIS CLAVARIAEFORMIS* SOLMS-LAUBACH

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(*Received 3 April 1940*)

In 1936 our attention was drawn by Prof. Ch. Dhéré of the University of Fribourg to a unique fungal colouring matter, long known as "mycoporphyrin", which is present in nature in *Penicilliopsis clavariaeformis* Solms-Laubach, a fungus peculiar to Java and found on the fallen and decaying fruits of *Diospyros macrophylla* Bl. [Solms-Laubach, 1887]. The whole fructification of this fungus is sulphur-yellow in colour, and Reinke [1887], in an accompanying paper, found that alcohol gives a yellow to orange extract which, on long standing, becomes purplish-red by transmitted light and shows a very striking orange-red fluorescence by reflected light. Reinke isolated from this extract red prisms which were not examined chemically but to which he gave the name mycoporphyrin since the substance gives an absorption spectrum which very closely resembles that of the true porphyrins. Other workers, e.g. Möller [1901], Hennings [1904] and Blochwitz [1931], have described investigations on the colouring matters of different species of *Penicilliopsis* which did not lead to any clearer ideas as to the chemical constitution of mycoporphyrin. Fischer & Niemann [1925] showed however by a series of reactions on an alcoholic extract of the naturally occurring fungus that mycoporphyrin, in spite of its absorption spectrum, cannot be a true porphyrin. The colouring matter itself was not isolated or analysed.

When we decided to commence work on the colouring matter of *P. clavariaeformis* it immediately became obvious that, in order to obtain sufficient material for a thorough chemical investigation, it would be necessary to work with laboratory cultures. It was found that the fungus grows quite well on the well-known synthetic Czapek-Dox, $NaNO_3$-glucose-mineral salts medium, and that if an orange extract be added to this medium and the $NaNO_3$ be replaced by ammonium citrate, approximately equivalent in nitrogen to the $NaNO_3$, luxuriant and typical growth is obtained. These cultures were incubated at 24° and it is important to note that they were grown *in the absence of light*. Solvent extraction of the fungus so obtained has led to the isolation of very considerable amounts—about 14 g. per 100 flasks—of an orange crystalline substance which we propose to name *penicilliopsin*. An account of our earlier work on penicilliopsin and its derivatives was given to the Society in 1938 [Oxford, 1938].

Penicilliopsin, M.P. about 330°, has the empirical formula $C_{30}H_{24}O_8$ and it contains 2 C-methyl groups as was shown by a Kuhn-Roth oxidation. It forms a diacetate and a *bis*phenylcarbamate, and analyses and molecular weight estimations on both these substances confirmed the empirical formula given. It reacts vigorously with diazomethane and diazoethane giving derivatives,

neither of which contains nitrogen, and corresponding respectively to a tetramethyl and diethyl ether. In neither case, however, could the alkoxyl groups presumably present be split off as alkyl iodide in a Zeisel estimation. On heating in air either alone or mixed with zinc dust penicilliopsin gives *Frangula*-emodin anthranol and, on distillation with zinc dust in hydrogen, it yields 2-methylanthracene. Hence penicilliopsin must be related to the polyhydroxyanthraquinones, a conclusion which is supported by the fact that it gives tetranitro-*Frangula*-emodin and nitrococcusic acid on oxidation with HNO_3.

Solutions of penicilliopsin in most organic solvents are yellow to orange in colour when freshly prepared, but on exposure to air and light undergo a series of colour changes which vary very much with different solvents. These colour changes involve two quite distinct reactions.

(*a*) *An oxidation process* dependent on the presence of atmospheric oxygen but not dependent on the presence of light. This process, which takes place very quickly in the dark in solutions of penicilliopsin in certain nitrogenous organic solvents, e.g. piperidine, or in neutral organic solvents such as ethanol or dioxane containing small amounts of organic nitrogenous bases, e.g. piperidine or brucine, results in the uptake of 3 atoms of oxygen per mol. of penicilliopsin and the formation of intensely purple non-fluorescent solutions. The oxidation product, which we propose to name *oxypenicilliopsin*, has the empirical formula $C_{30}H_{20}O_9$ and forms purplish-black aggregates which do not melt at 360° and which give a tetra-acetate on acetylation with acetic anhydride containing conc. H_2SO_4. Oxypenicilliopsin gives colour reactions which are quite different from those given by penicilliopsin and, in striking contrast to penicilliopsin, does not give tetranitroemodin on oxidation with conc. HNO_3.

(*b*) *A photochemical process*, independent of the presence of oxygen but dependent on the presence of light. This process results in the conversion of oxypenicilliopsin into an isomeric compound which we propose to name *irradiated oxypenicilliopsin*. When an alcoholic solution of oxypenicilliopsin, deep purple in colour and non-fluorescent, is exposed to bright daylight either under aerobic or anaerobic conditions the original deep purple colour soon begins to fade, a fiery red fluorescence begins to develop and irradiated oxypenicilliopsin separates in chocolate brown needles which do not melt up to 370°. Irradiated oxypenicilliopsin has the empirical formula $C_{30}H_{20}O_9$ and is, in our opinion, the pure substance which is responsible for the earlier conceptions of mycoporphyrin. A careful study of previous literature on mycoporphyrin leads to the conclusion that what has been previously understood as mycoporphyrin was, in reality, a mixture, in varying amounts, of penicilliopsin, oxypenicilliopsin and irradiated oxypenicilliopsin. Since none of these substances contains nitrogen it is obvious that, quite apart from the previously quoted evidence of Fischer & Niemann [1925], mycoporphyrin cannot be a true porphyrin and hence in agreement with Dhéré & Castelli [1937] we suggest that the misnomer mycoporphyrin be dropped from the literature. We regard this matter as one of some importance since, to quote Dhéré & Castelli [1937], "les dermatologistes désignent maintenant parfois sous le nom de 'mycoporphyrines' les porphyrines vraies extraites des champignons microscopiques agents des 'mycoses' cutanées".

There is present in the petals and stamens of St John's Wort, *Hypericum perforatum*, a red colouring matter known as hypericin or hypericum red, the photodynamic properties of which are of particular interest in comparative pathology. It has been shown by a number of workers [Ioannides, 1930; Hausmann, 1932; Quin, 1933; Horsley, 1934] that the photodynamic action of hypericin is responsible for a pathological condition caused by light and known

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as hypericismus. Grazing animals which, after feeding on St John's Wort, are exposed to bright sunlight, show a characteristic photosensitization and in extreme cases die. Fischer & Hess [1930] compared the absorption spectra of crude alcoholic extracts of St John's Wort and naturally occurring *P. clavariaeformis* and concluded that hypericin is identical with mycoporphyrin. The same conclusion was reached by Dhéré [1933] who examined the fluorescence spectra of similar solutions. Later, Dhéré & Castelli [1937] examined the fluorescence spectrum at ordinary temperatures of a specimen of our oxypenicilliopsin and the same workers [1938] compared, at the temperature of liquid air, the fluorescence spectrum of our oxypenicilliopsin with that of an alcoholic extract of the petals of *Hypericum perforatum*. Finally, Dhéré & Castelli [1939] compared both the absorption and fluorescence spectra, at ordinary temperatures and in liquid nitrogen, of our irradiated oxypenicilliopsin with those of a purified specimen of hypericin from St John's Wort kindly given to us by Drs Mackinney and Pace of the University of California [Mackinney & Pace, 1939]. The continental workers could detect no differences between the very remarkable spectra of irradiated oxypenicilliopsin and hypericin, and concluded that they must either be the same substance or be very closely related to each other.

Our own purely chemical findings definitely exclude the identity of irradiated oxypenicilliopsin (mycoporphyrin) with hypericin though they certainly point to a close relationship between them. A series of comparative colour reactions, carried out early in 1939 on pure irradiated oxypenicilliopsin and Mackinney & Pace's hypericin, while showing a very close resemblance in many reactions, gave in one or two instances decisive differences.

So far as we are aware, the only work on the chemical constitution of hypericin published before 1939 is that of Cerny [1911] who assigned to it the empirical formula $C_{16}H_{10}O_5$ and suggested that it might belong to the flavones. A very recent publication by Brockmann *et al.* [1939] however records preliminary results on the chemical constitution of hypericin which support our conclusion that hypericin is not identical with irradiated oxypenicilliopsin. These workers assign the empirical formula $C_{28}H_{16}O_8$ to hypericin (M.P. about 330°), describe a hexabenzoate and advance the tentative suggestion that it may be a hexahydroxy derivative of *meso*dianthrone.

Our experimental work has not yet progressed sufficiently far to justify the expression of more than a very tentative opinion of the probable molecular constitution of penicilliopsin and its oxidation products. It may well be, however, that penicilliopsin ($C_{30}H_{24}O_8$) is a dimeride of emodin anthranol ($C_{15}H_{12}O_4$), which was obtained from penicilliopsin by dry distillation, in which case penicilliopsin may be regarded as a polyhydroxy derivative of a reduced *meso*dimethyldianthrone. We are of the opinion, however, that oxypenicilliopsin ($C_{30}H_{20}O_9$) and irradiated oxypenicilliopsin ($C_{30}H_{20}O_9$) are not dianthrones but are more probably derivatives of helianthrone or of naphthadianthrone with which they have many properties in common particularly in the colour of the crystals and in their colour reactions with many reagents.

The work on the chemical constitution of penicilliopsin is being continued.

EXPERIMENTAL

History and description of culture

We are indebted to our colleague, Mr G. Smith, for the information given in this section.

Penicilliopsis clavariaeformis Solms-Laubach was first isolated by H. Grafen zu Solms-Laubach [1887] from fallen, rotting fruits of *Diospyros macrophylla* Bl., in the Buitenzorg Botanical Gardens, Java. In its natural habitat it forms clusters of globose perithecia, similar to those of *Aspergillus glaucus* and some species of *Penicillium*, and also conidial fructifications in the form of *Clavaria*-like masses of sulphur-yellow coremia, the ultimate elements of which are essentially similar to the conidiophores of typical *Penicillia*.

The culture used throughout this work, L.S.H.T.M. Cat. No. X 12, was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland, in June 1936 and was derived from a naturally occurring strain originating in Java. In cultural experiments on a variety of solid culture media and at various temperatures, normal coremia were formed in almost all cases but under no conditions was production of perithecia observed. The best of the media tried was orange extract agar, on which at 24° growth is very rapid, with formation of thick clusters of coremia several cm. in length, sulphur-yellow at first, then greenish-yellow and finally brown in old cultures.

Cultural conditions

P. clavariaeformis has been grown here on each of the following liquid media (*a*), (*b*) and (*c*). The mould grew quite well on both media (*a*) and (*b*) but gave a much more luxuriant and typical growth on medium (*c*). Penicilliopsin was isolated from the dried fungus grown on each of these media but as medium (*c*) gave a much better yield of pure penicilliopsin than either (*a*) or (*b*), it was used for the preparation of the colouring matter in quantity.

(*a*) Unhopped beer wort, sp. gr. 1·04.

(*b*) Czapek-Dox solution. Glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; distilled water to 1000 ml.

(*c*) Orange extract medium. Glucose, 50 g.; triammonium citrate, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; extract of one orange; *N* HCl, sufficient to adjust the *p*H to 4·5 (usually about 6·0 ml.); distilled water to 1000 ml. For the preparation of the orange extract, fair sized oranges, one for each litre of finished medium, were minced and the pulp boiled with water, 500 ml. per orange. The extract was strained through muslin and the pulp was further extracted with boiling water, 250 ml. per orange. The combined extracts were cleared by filtration through filter paper pulp. The luxuriance of growth varied somewhat with the variety of orange used, the best results being obtained with Brazilian oranges when in season.

For the preparation of penicilliopsin in quantity, 350 ml. of medium (*c*) were placed in each of 100 1-l. conical flasks, plugged with cotton wool, which were sterilized by steaming for 1 hr. on each of 3 consecutive days. Each flask was then inoculated with a spore suspension in sterile distilled water of a mature culture of *P. clavariaeformis*, L.S.H.T.M. Cat. No. X 12, on a beer-wort agar slope, 4 flasks being sown from 1 slope. The flasks were incubated at 24° in the dark until the glucose content of the filtered metabolism solution, as measured by the polarimeter, was almost zero, the average incubation period being 14–16 days. The details of a number of typical experiments on media (*a*), (*b*) and (*c*) are given in Table 1.

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Isolation and purification of penicilliopsin

At the end of the incubation period the mould was separated from the metabolism solution by straining through butter muslin, washed well with water, dried in a vacuum oven at 50–60° and weighed. The strained metabolism solution contains large amounts of spores of the fungus which, in one experiment with 100 flasks of medium (*c*), were separated, washed and dried (wt. 18 g.). Since solvent extraction of the dried spores showed that they contain practically no penicilliopsin they were discarded in subsequent experiments. The dried fungus was finely ground in a coffee mill and thoroughly extracted with light petroleum, B.P. 40–50°, in a large Soxhlet apparatus. This treatment removed small amounts of a semi-solid fatty material containing practically no penicilliopsin. The light petroleum-extracted fungus was air-dried and then exhaustively extracted with ether, 5–6 working days being necessary for complete extraction. Crude penicilliopsin separated from the ether extracts in brownish-red solid crusts which were collected daily. This crude material was purified by two crystallizations from boiling dioxane (10–12 ml. per g. of crude pigment) and was thus obtained in small, light orange, stout rectangular prisms. Details of yields obtained in a number of representative experiments are given in Table 1. The losses incurred in crystallizing penicilliopsin from dioxane were disappointingly large, and the fact that these losses are due to oxidation and irradiation was only realized at a late stage. No doubt they could be much reduced and the recovery yield of pure penicilliopsin be greatly increased by keeping all dioxane solutions and even ether solutions in the dark in almost full flasks, which should be cooled quickly and then corked immediately to prevent access of air. The only solvents in which penicilliopsin is not fairly rapidly oxidized are unfortunately those in which it is only very sparingly soluble even at the boiling point, i.e. chloroform and toluene. There was no evidence, at any time, of the presence, in the ether extracts of the fungus, of any crystalline colouring matter other than penicilliopsin.

Table 1

Medium	Number of flasks	Incubation period days	Glucose by polarimeter %	Dry wt. of fungus g.	Wt. of crude penicilliopsin g.	Wt. of pure penicilliopsin crystallized from dioxane g.
(*a*)	2	68	—	14·0	—	0·13
(*b*)	75	53	Nil	268	3·0	1·68
(*c*)	100	14	0·1	451	33·2	14·0
(*c*)	100	14	0·1	450	34·0	13·9

Penicilliopsin

(1) *General properties.* Penicilliopsin is practically insoluble in light petroleum, only slightly soluble in boiling toluene or chloroform, and rather more soluble in boiling ethanol or ethyl acetate. It crystallizes most readily from hot dioxane, in which it is moderately soluble, in microscopic, orange, stout rectangular or occasionally hexagonal prisms. The colour of the specimens prepared in this way varied from orange-yellow through orange-red to brownish-red, possibly on account of varying crystal sizes, but more probably because the more darkly coloured specimens were contaminated with small amounts of an oxidation product (see p. 799). When crystallized from ethanol it separated in very minute crystals of similar shape which were invariably orange-yellow in colour.

Penicilliopsin, crystallized from dioxane, contains water and dioxane of crystallization which are lost on heating strongly, the substance becoming yellowish-brown in colour at 200° and melting sharply at about 330° without effervescence to a purplish-black liquid which shows a definite meniscus before 340°. The M.P. varies somewhat with the rate of heating. A specimen crystallized from ethanol behaved similarly on heating.

An alcoholic solution gives a dark, olive-brown colour with $FeCl_3$. Penicilliopsin dissolves in cold conc. H_2SO_4 with a yellowish-brown colour, which quickly changes to green on warming and then develops a strong dull red fluorescence.

Penicilliopsin is insoluble in aqueous sodium acetate, slowly soluble in Na_2CO_3 and much more readily soluble in NH_4OH and very dilute NaOH and KOH giving brown solutions in each case which, on long standing or boiling for a short time become brownish-red with Na_2CO_3, brownish-purple with NH_4OH and purple with NaOH or KOH. The characteristic behaviour with organic bases is described later (see p. 799).

(2) *Analysis of penicilliopsin.* Difficulties were experienced in arriving at an empirical formula for penicilliopsin since the solvents which are most suitable for its purification, especially dioxane, cling tenaciously to the substance. It is too slightly soluble in any solvent to allow an accurate mol. wt. estimation to be made but this difficulty was overcome by determining the mol. wt. of some of its derivatives (see p. 796).

Specimens crystallized from three different solvents were analysed with the following results.

(1) Crystallized from toluene containing a little acetic acid and dried to constant weight in a high vacuum at 160–170°. (Found: C, 69·87, 70·04; H, 4·66, 4·54; N, nil; CH_3O, nil; $C_{30}H_{24}O_8$ requires C, 70·28; H, 4·72%.)

(2) Crystallized from dioxane. (*a*) Air-dried. (Found: C, 67·12, 67·19; H, 5·24, 5·28. $C_{30}H_{24}O_8$, $\frac{1}{2}H_2O$, $C_4H_8O_2$ requires C, 66·95; H, 5·43%.) (*b*) Dried to constant weight at 100–110° in a high vacuum. (Found: C, 68·14, 68·20; H, 4·94, 5·15; side chain CH_3, 4·82. $C_{30}H_{24}O_8$, $C_4H_8O_2$ requires C, 67·98; H, 5·37; $2CH_3$ as $CH_3.C$, 5·00%.)

(3) Crystallized from ethanol. (*a*) Specimen from orange extract medium dried to constant weight in a high vacuum at 100°. (Found: C, 66·99, 66·75; H, 5·09, 5·20; CH_3O, nil. $C_{30}H_{24}O_8$, $1\frac{1}{2}H_2O$ requires C, 66·76; H, 5·04%.) (*b*) Specimen from Czapek-Dox medium and dried as 3*a*. (Found: C, 67·00, 66·89; H, 5·09, 5·23; CH_3O, nil. $C_{30}H_{24}O_8$, $1\frac{1}{2}H_2O$ requires C, 66·76; H, 5·04%.)

(3) *Derivatives of penicilliopsin.* Penicilliopsin does not react with 2:4-dinitrophenylhydrazine hydrochloride in hot aqueous dioxane solution and was recovered unchanged when its dioxane solution was shaken with hydrogen in presence of a palladium-norite catalyst. Attempts to make the *p*-xenylcarbamate, the *p*-chlorophenylcarbamate, the *p*-bromobenzoate, the ethyl ester by the Fischer-Speier method, the methyl derivative by methylation with methyl sulphate and alkali in presence of $Na_2S_2O_4$, and the acetyl derivative by the acetic anhydride-sodium acetate method all gave uncrystallizable products, or crystalline products which were artefacts. It is remarkable that *p*-bromobenzoyl chloride has practically no action on penicilliopsin even at 160–170°.

(*a*) *Penicilliopsin* bis*phenylcarbamate.* Penicilliopsin (1·5 g.), crystallized from dioxane and dried at 140° in a high vacuum, was refluxed with a large excess of phenyl *iso*cyanate (5 g.) for 1·5 hr. in an oil bath at 175°. After about 1 hr. the substance had completely dissolved to an almost black solution. After cooling, the solution was diluted with much light petroleum and the greyish-brown precipitate was collected (2·0 g.). This was repeatedly extracted with

boiling benzene, the first red extract being rejected and the yellow material separating from the second and third extracts was recrystallized from boiling benzene in which the product is sparingly soluble. *Penicilliopsin* bis*phenylcarbamate* forms small, yellow prisms (parallelepipeds), M.P. 238–240°, with effervescence and blackening. (Found, on material dried to constant wt. in a high vacuum at 140°: C, 70·10, 70·25; H, 4·50, 4·66; N, 3·91, 3·67 %. Mol. wt. (cryoscopic in dioxane), 771. $C_{44}H_{34}O_{10}N_2$, i.e. the *bis*phenylcarbamate of $C_{30}H_{24}O_8$, requires C, 70·36; H, 4·57; N, 3·73 %. Mol. wt. 750.) The substance is readily soluble in cold dioxane, and in pyridine to give a deep red solution which exhibits a strong red fluorescence after standing in daylight for some time. It dissolves quickly but not instantly in aqueous KOH to yield a deep brown solution, hydrolysis to penicilliopsin probably taking place.

(*b*) *Diacetylpenicilliopsin.* Penicilliopsin (0·15 g.), treated with boiling acetic anhydride (6 ml.), dissolved in about 5 min. The red solution was boiled for a further 2 min., and on cooling deposited orange-yellow crystals (0·05 g.) which were crystallized first from ethyl acetate and then from benzene. *Diacetylpenicilliopsin* forms orange-yellow rectangular prisms which darken on heating and slowly decompose above 280°. (Found, on material dried at 110° in a high vacuum: C, 68·60, 68·60; H, 4·47, 4·42; hydrolysable CH_3CO, by alkaline hydrolysis, 14·5 %; side-chain CH_3, by Kuhn-Roth oxidation, 8·9 %; mol. wt. in camphor, 636, 614; $C_{34}H_{28}O_{10}$, i.e. the diacetate of $C_{30}H_{24}O_8$, requires C, 68·45; H, 4·73; $2CH_3CO$, 14·4; $4CH_3$ as CH_3.C, 10·1 %; mol. wt. 596.) The substance is much more soluble in the usual organic solvents, with the exception of ethanol and dioxane, than is the parent compound. Its reactions are exactly the same as those of the parent substance except that it is not immediately soluble in aqueous Na_2CO_3. The acetyl groups are very stable to acid hydrolysis.

(4) *Action of diazomethane on penicilliopsin.* Penicilliopsin (0·4 g.) was treated with a large excess of ethereal dizaomethane. There was an immediate, vigorous effervescence and a yellow, flocculent precipitate later formed which was collected and extracted with much boiling glacial acetic acid. The residue was crystallized first from nitrobenzene and then from boiling toluene in which it is very sparingly soluble. The product formed small orange-yellow platelets (parallelepipeds) which darkened above 300° and melted indefinitely at 340–360° to a black liquid. (Found on material dried to constant wt. in a high vacuum at 190–210°: C, 71·91, 71·85; H, 5·51, 5·36; N, nil; CH_3O, nil. $C_{34}H_{32}O_8$, i.e. $C_{30}H_{24}O_8+4CH_2$, requires C, 71·80; H, 5·55 %.) The product gives a brown solution in cold conc. H_2SO_4 but is insoluble in NaOH except on very long standing when a brown solution is obtained. It gives an immediate reddish-brown colour with pyridine and the solution soon exhibits a red fluorescence on exposure to daylight.

(5) *Action of diazoethane on penicilliopsin.* This product was prepared as described above except that diazoethane was used. The product, which is much more soluble in toluene than the corresponding methyl derivative, was crystallized from dioxane in which it is very sparingly soluble in the cold. It formed minute yellow square tablets frequently having rounded corners and melted indefinitely about 310° to a dark red liquid. (Found on material dried at 140° in a high vacuum: C, 71·74, 71·66; H, 5·58, 5·48; N, nil, C_2H_5O, nil. $C_{34}H_{32}O_8$, i.e. $C_{30}H_{24}O_8+2C_2H_4$, requires C, 71·80; H, 5·55 %.) The product is very slowly soluble in dilute NaOH to give a brown solution. It gives an immediate reddish brown colour with pyridine and the solution soon exhibits a red fluorescence on exposure to daylight.

(6) *Action of heat on penicilliopsin. Formation of* Frangula-*emodin anthranol.* An intimate mixture of pure penicilliopsin (0·2 g.) and zinc dust (3·5 g.) was placed in the closed end of a small bent test tube the open end of which was connected to a cooled receiver. The mixture was strongly heated when a yellowish-brown sublimate together with a brown liquid, which later solidified, collected in the cooler limb. The combined products from a number of similar experiments (about 25 mg. from 2 g. of penicilliopsin) were crystallized from ethyl acetate in small pale yellow prisms which blackened and softened above 260°. (Found: C, 70·20, 70·21; H, 4·98, 4·73 %. $C_{15}H_{12}O_4$ requires C, 70·28; H, 4·72 %.)

The substance was shown to be *Frangula*-emodin anthranol, 4:5:7-trihydroxy-2-methylanthranol (9 or 10), by direct comparison with an authentic specimen prepared by the reduction of physcion (4:5-dihydroxy-7-methoxy-2-methylanthraquinone) with conc. HI, sp. gr. 1·7. The two specimens crystallized from ethyl acetate in an exactly similar way and showed the same behaviour on heating, either alone or in admixture. Their colour reactions with the following reagents were also identical. (*a*) Dilute NaOH gave a solution which became purple on shaking with air owing to oxidation to emodin, 4:5:7-trihydroxy-2-methylanthraquinone. (*b*) Conc. ammonia (sp. gr. 0·880) gave a solution which is dark red by transmitted light and purple in thin layers. (*c*) Conc. H_2SO_4 gave a solution which became green on standing and showed a deep green fluorescence on heating. (*d*) A solution in pyridine containing some piperidine did not develop a strong red fluorescence on standing in daylight, cf. penicilliopsin (p. 799).

A similar sublimate of emodin anthranol was formed in smaller amount when penicilliopsin was heated alone or mixed with kieselguhr.

Oxypenicilliopsin (see p. 799) gave a trace of a yellow sublimate, possibly emodin anthranol, when similarly heated with zinc dust, but irradiated oxypenicilliopsin (see p. 801) gave no sublimate at all under these conditions.

(7) *2-Methylanthracene from penicilliopsin.* An intimate mixture of pure penicilliopsin (4 g.) and zinc dust (80 g.) was distilled at a red heat for 1 hr. in a slow stream of hydrogen. The yellowish-brown sublimate (about 0·05 g.), after resublimation at 200–250° at atmospheric pressure was crystallized from aqueous ethanol giving almost colourless plates with a bluish lustre, M.P. 196°, raised to 197–198° by a second sublimation. (Found: C, 93·64; H, 6·45 %. $C_{15}H_{12}$, i.e. methylanthracene, requires C, 93·71; H, 6·30 %. $C_{14}H_{10}$, i.e. anthracene, requires C, 94·34; H, 5·66 %.) The M.P. of the substance was not depressed on admixture with an authentic specimen of 2-methylanthracene and the two hydrocarbons, after sublimation, were also identical in appearance, viz. shining almost colourless plates with a fine green fluorescence. Other experiments on similar lines confirmed the formation of 2-methylanthracene but failed to improve the yield.

(8) *Oxidation of penicilliopsin with nitric acid. Isolation of tetranitro-*Frangula-*emodin, nitrococcusic acid and oxalic acid.* Many experiments were carried out in order to obtain the maximum yield of recognizable oxidation products and the following method was finally adopted.

Penicilliopsin (3·0 g., crystallized from dioxane and air-dried) was suspended in conc. HNO_3 (25 ml., sp. gr. 1·4) and evaporated to dryness in a porcelain dish on the steam bath with frequent stirring, the initial reaction being very vigorous. The dark gummy residue was dissolved in hot glacial acetic acid (10 ml.) and the yellow crystals which formed on standing were separated (1·05 g.). The filtrate was freed from acetic acid *in vacuo* over solid KOH and the residue was re-treated with HNO_3 (10 ml.) and re-extracted with acetic acid yielding a further 0·78 g. of crystalline material. A third treatment with HNO_3 (5 ml.) of the oily

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residue from these crystals gave a final crop of hard, brown, resinous material (0·35 g.). The total yield of solid oxidation products (2·18 g.) was fractionated as follows.

(*a*) *Separation and identification of tetranitro*-Frangula-*emodin* (*4:5:7-trihydroxy-1:3:6:8-tetranitro-2-methylanthraquinone*). The combined products were dissolved in boiling glacial acetic acid (18 ml.) and the yellow crystalline material (0·43 g., M.P. 290°) separating after standing for 2 days was crystallized from dry benzene-light petroleum. M.P. 296°. The product was shown to be tetranitro-*Frangula*-emodin by direct comparison with a synthetic specimen prepared from *Frangula*-emodin by the method described by Oesterle [1911]. The two specimens had the following analyses. (Found: (*a*) on specimen from penicilliopsin, C, 40·17, 40·05; H, 1·62, 1·50; N, 12·6, 12·3 %; (*b*) on specimen from *Frangula*-emodin, C, 39·98, 40·11; H, 1·67, 1·86; N, 12·0, 12·2 %. $C_{15}H_6O_{13}N_4$ requires C, 40·00; H, 1·34; N, 12·4 %.) The oxidation product, on further purification, melted at 298°, the synthetic product at 302°, and a mixture of the two at 298°. They had each the same crystalline form—slender, orange prismatic rods—and they gave identical reactions as follows: (1) a deep orange-red solution in water; (2) dilute aqueous NH_4OH, a violet solution; (3) conc. NH_4OH, a deep purplish-red solution; (4) dilute aqueous Na_2CO_3, a deep reddish-brown solution; (5) 2*N* NaOH, almost insoluble, the solid becoming almost black; (6) conc. H_2SO_4, brownish-yellow solution; (7) dilute boiling aqueous sodium sulphide, deep violet solution.

(*b*) *Separation of oxalic acid as calcium oxalate.* The acetic acid filtrate from the first crystallization of the combined oxidation products was evaporated to dryness over solid KOH. The light brown solid residue (1·75 g.) contained a considerable amount of colourless material and an estimation, on a part of it (0·2843 g.), of the oxalic acid present gave calcium oxalate corresponding to 0·554 g. of anhydrous oxalic acid in the total residues.

(*c*) *Separation and identification of nitrococcusic acid* (*2:4:6-trinitro-5-hydroxy-3-methylbenzoic acid*). The remainder of the solid from the acetic acid filtrate (1·47 g.) was treated with boiling water (8 ml.), filtered from a little insoluble coloured material (0·01 g.) and kept at 0°, when hydrated oxalic acid (0·05 g.) together with a trace of tetranitroemodin separated. The aqueous filtrate was evaporated to dryness *in vacuo* and the residue crystallized from glacial acetic acid (4 ml.). Long pale yellow needles separated (0·35 g.), M.P. 206–208° decomp., if heated slowly. The substance explodes if suddenly plunged into an oil bath at 250°. (Found on air-dried material: C, 32·01, 31·98; H, 2·58, 2·53; N, 13·4, 13·3 %. $C_8H_5O_9N_3,H_2O$ requires C, 31·48; H, 2·31; N, 13·7 %.) The substance is readily soluble in water giving a yellow, strongly acidic solution. It is undoubtedly nitrococcusic acid (2:4:6-trinitro-5-hydroxy-3-methylbenzoic acid) [Kostanecki & Niementowski, 1885] which is known to crystallize with 1 H_2O [Charles *et al.* 1933]. The same compound was obtained by the action of HNO_3, sp. gr. 1·4, on *Frangula*-emodin and melted at 206–208° decomp., alone or mixed with the product from penicilliopsin.

When penicilliopsin was oxidized with dilute HNO_3, sp. gr. 1·2, at 100° the main oxidation product was nitrococcusic acid, only traces of tetranitroemodin being formed while, under the same conditions, emodin yielded mainly tetranitroemodin with only traces of nitrococcusic acid, and tetranitroemodin is almost unaffected by HNO_3 of this concentration. It is therefore probable that the nitrococcusic acid formed when penicilliopsin is oxidized with HNO_3 of sp. gr. 1·2 arises not from emodin or tetranitroemodin, but rather from the direct breakdown of the penicilliopsin molecule itself.

Oxypenicilliopsin

Solutions of penicilliopsin in different organic solvents, when exposed to air and light, undergo a series of colour changes which vary with the nature of the solvent used. These changes involve, as stated in the introduction, two separate and distinct reactions; thus a saturated solution of penicilliopsin in chloroform is bright yellow in colour and remains unchanged when exposed to air and light for a week, no purple colour or fluorescence being developed. On the other hand an alcoholic, ethereal or dioxane solution becomes progressively darker under these conditions and a strong red fluorescence is slowly developed; if a saturated alcoholic solution is used a slight purple deposit is formed on long standing.

(*a*) *The oxidation process.* The oxidation of penicilliopsin to oxypenicilliopsin takes place very slowly in neutral organic solvents, e.g. ethanol, ether, dioxane etc., but increases in rapidity on the addition to these solvents of certain organic bases and proceeds with great rapidity when solutions of penicilliopsin in these basic organic solvents are exposed to air in the dark.

The most effective base for this purpose is piperidine, though other bases of strength comparable with that of piperidine, e.g. brucine, cinchonine, methylamine and ethylamine, were almost equally effective. Pure pyridine is not a strong enough base to bring about a quick oxidation, but samples of commercial pyridine containing piperidine, or pure pyridine to which a small amount of piperidine is added, are quite effective. The following bases are ineffective: quinoline, aniline, dimethyl- and diethyl-aniline, α-naphthylamine, caffeine and *m*-aminobenzoic acid (the last three in alcoholic solution).

Up to the present we have not been able to oxidize penicilliopsin to oxypenicilliopsin rapidly except by the use of a strong *nitrogenous* base. Thus while ammonia and hydrazine behave like the strong organic bases, KOH behaves in a different manner. If a few drops of alcoholic KOH are added to an alcoholic solution of penicilliopsin a purple-violet colour develops on standing but no fluorescence appears on exposure to daylight. If excess of alcoholic HCl is now added and the precipitated KCl is separated by filtration the filtrate is merely yellowish-brown in colour and develops only a weak red fluorescence on exposure to daylight. In a corresponding experiment with brucine, piperidine or methylamine, the reddish-purple colour remained on addition of excess of alcoholic HCl and a very strong red fluorescence soon developed when either the original or the acidified solution was exposed to daylight.

Finally the characteristic oxidation cannot be carried out in aqueous solution and, indeed, when carried out in alcoholic solution, absolute alcohol must be used since the addition of only a small amount of water prevents the development of the characteristic deep reddish-purple colour.

The following experiment proves conclusively that the conversion of penicilliopsin into oxypenicilliopsin does in fact necessitate the uptake of atmospheric oxygen but is independent of light. A few ml. of pyridine containing about 5% of piperidine were placed in a long, wide specimen tube into which was inserted a smaller narrow tube filled with penicilliopsin and so arranged that the smaller tube rested with its mouth well above the level of the pyridine-piperidine mixture. The two tubes were carefully inserted into a Büchner filtration flask to which was added some freshly prepared alkaline pyrogallol. The air in the Büchner flask was then removed as completely as possible by a vacuum pump and the evacuated flask was sealed from the air. After standing for some time to remove the last traces of oxygen the flask was tilted so that a small amount of penicilliopsin was dissolved by the pyridine-piperidine mixture. The apparatus was

then kept in the dark for several hours when the penicilliopsin solution had become merely brownish-red in colour with no trace of a purple tint. It was exposed to daylight for some time but no perceptible fluorescence developed. Air was now admitted and the flask immediately placed in the dark. The solution quickly became much darker in colour and a purple tint was evident at the air-liquid interface. After standing overnight in the dark the solution was a deep reddish-purple by transmitted light and, on exposure to daylight, quickly developed a blood red fluorescence.

(*b*) *Measurement of oxygen uptake in the oxidation of penicilliopsin to oxypenicilliopsin.* The measurement of the amount of oxygen absorbed in the oxidation of penicilliopsin to oxypenicilliopsin was carried out in the Barcroft differential apparatus. Pure penicilliopsin, crystallized from dioxane and air-dried, was used and in both the experiments recorded dioxane was employed as solvent with piperidine as the organic base in the one experiment and brucine in the other. At the end of each experiment a good deal of oxypenicilliopsin had separated from the solution. It is evident from the experimental details given below that the oxidation proceeds according to the equation

$$C_{30}H_{24}O_8 + 3O \rightarrow C_{30}H_{20}O_9 + 2H_2O.$$

Exp. 1. Left-hand vessel contained penicilliopsin (0·00883 g.), dioxane (3·0 ml.) and piperidine (0·015 ml.).

Right-hand vessel contained dioxane (3·0 ml.) and piperidine (0·015 ml.). The absorption of oxygen was virtually complete in 3 hr. Vol. of oxygen absorbed, 420·3 μl. Theor. for 3 atoms of oxygen per mol. of penicilliopsin, 487 μl.

Exp. 2. Left-hand vessel contained penicilliopsin (0·01014 g.), dioxane (3·0 ml.) and brucine (0·02 g.).

Right-hand vessel contained dioxane (3·0 ml.) and brucine (0·02 g.). The absorption of oxygen was much slower than in exp. 1 but was complete in 26 hr. Vol. of oxygen absorbed, 587·8 μl. Theoretical, 559·4 μl.

(*c*) *Preparation and properties of oxypenicilliopsin.* Pure penicilliopsin (1 g.) was dissolved in a mixture of pyridine (100 ml.) and piperidine (8 ml.) contained in a 1 l. conical flask the neck of which was loosely corked with a grooved cork to allow access of air. The mixture was immediately placed in the dark and kept there, with occasional shaking, for 4 days. The permanganate-coloured solution so obtained was poured into an excess of well cooled and stirred dilute HCl and, after 2 days in a dark cold room, the dark purple flocculent precipitate was collected, washed and dried (*ca.* 1·0 g.). The crude oxidation product proved exceptionally difficult to obtain in a satisfactory crystalline form. It separated from hot dioxane, in which it is less readily soluble than penicilliopsin, in amorphous aggregates which were sparingly soluble in hot glacial acetic acid. It separated from this solvent in small aggregates which were not definitely crystalline under the microscope. Finally it separated from nitrobenzene or ethanol in large purplish-black masses, the edges of which appeared to have a crystalline structure. The product from nitrobenzene did not melt at 360° nor did it sublime appreciably at 240–250° in a high vacuum. (Found, on material from nitrobenzene dried to constant weight in a high vacuum at 200–210°: C, 68·77, 68·85; H, 3·84, 3·81; N, nil. $C_{30}H_{20}O_9$ requires C, 68·68; H, 3·85%.)

Oxypenicilliopsin dissolves sparingly in all the usual organic solvents with the formation of a deep reddish-purple colour, which is stable in the dark but fades in daylight with the development of an intense blood-red fluorescence. A basic solvent is not necessary for the development of the fluorescence; indeed it is best developed in hydrocarbon solvents like benzene or toluene. Oxypeni-

cilliopsin is insoluble in aqueous Na_2CO_3 but dissolves in NaOH to give a deep blue solution which becomes green on standing for some time and deposits a greyish-green precipitate after long standing, the supernatant liquid being pale purple. It dissolves in cold conc. H_2SO_4 giving a deep blue solution which becomes green on standing with the development of a striking dull red fluorescence; the original deep blue solution becomes green on the addition of solid $NaNO_2$ and pink on pouring into water. A freshly prepared alcoholic solution gives the following reactions: no marked change on the addition of aqueous $NaHCO_3$ or Na_2CO_3; an intense reddish-purple with a trace of aqueous ammonia; a bluish-purple colour with $NiSO_4$ solution, with precipitate on standing; solid MgO is coloured light blue and solid CaO a deep blue becoming green on standing.

It is remarkable that oxypenicilliopsin, on treatment with HNO_3, sp. gr. either 1·2 or 1·4, failed to give either tetranitroemodin or nitrococcusic acid, cf. the action of HNO_3 on penicilliopsin, p. 797.

(*d*) *Acetyl* (*probably tetra-acetyl*) *derivative of oxypenicilliopsin.* A solution of oxypenicilliopsin (0·5 g.) in acetic anhydride (30 ml.) containing a few drops of conc. H_2SO_4 was boiled for 5 min., cooled and poured on to crushed ice. The brownish-red precipitate was collected, washed and dried. It separated from a mixture of acetic acid (2 parts) and acetic anhydride (1 part) in minute, light orange-red, flat rods, M.P. 308–310° decomp. after darkening above 280°. (Found, on material dried to constant wt. in a high vacuum at 180°: C, 66·18, 66·10; H, 3·81, 3·94 %; mol. wt. cryoscopic in dioxane, 698·5. $C_{38}H_{28}O_{13}$, i.e. $C_{30}H_{16}O_5.(O.CO.CH_3)_4$, requires C, 65·89; H, 4·07 %; mol. wt. 692). Attempts to estimate accurately the acetyl content were not successful.

The acetyl derivative is only slowly soluble in cold NaOH. On warming, however, a deep violet solution results which changes to a deep pure green overnight. Its behaviour with conc. H_2SO_4 is exactly the same as that of oxypenicilliopsin. Solutions of the acetyl derivative in neutral organic solvents, e.g. ethyl acetate, dioxane, at first orange in colour, rapidly darken on exposure to bright daylight, but not if kept in the dark. There is no development of any fluorescence in daylight but a fluorescence develops if piperidine is added, probably owing to slow removal of acetyl groups.

Irradiated oxypenicilliopsin

Conversion of oxypenicilliopsin into irradiated oxypenicilliopsin. A cold saturated alcoholic solution of oxypenicilliopsin, prepared by refluxing an excess (1 g.) of the latter with absolute alcohol (250 ml.) for 10 min., filtering hot, keeping the filtrate overnight in the dark and then refiltering, was distributed in 100 6-in. test tubes, which were then left in an inclined position in front of a north window on a bright day in March 1939. After about 3 hr. irradiation, the original purple colour had visibly begun to fade, a fiery red fluorescence developed and chocolate-brown needles began to separate near the surface. After 2 days this crystalline product was collected, washed with ethanol and dried (0·20 g.). The substance was practically insoluble in boiling ethanol and was purified by recrystallization from dioxane. It had no M.P. and seemed to suffer no change, except a loss of crystalline shape, on heating to 370°. (Found, on material dried at 100–110° in a high vacuum: C, 68·84, 68·69; H, 4·00, 3·85; CH_3O, nil. $C_{30}H_{20}O_9$ requires C, 68·68; H, 3·85 %.)

No oxidation is therefore involved in the conversion of oxypenicilliopsin into irradiated penicilliopsin. This was further proved by sealing off an evacuated tube containing a cold, saturated alcoholic solution of oxypenicilliopsin and then submitting it to the action of daylight when the separation of chocolate-brown

needles and the development of a strong red fluorescence took place exactly as before when in the presence of air.

The general colour reactions of irradiated oxypenicilliopsin are given in the next section where they are compared, side by side, with those of hypericin. Other points of interest in irradiated oxypenicilliopsin are the following: it is very sparingly soluble in most neutral organic solvents but the solutions exhibit immediately the characteristic fiery red fluorescence associated with hypericin and "mycoporphyrin"; the red fluorescence persists even in acetic anhydride solution, but disappears on the addition of a trace of conc. H_2SO_4, the solution becoming redder and, on standing, bluer in tone and finally taking on a mauve tint. The reaction with dilute NaOH (see below) recalls that of certain naturally occurring 2-hydroxyphenanthraquinones described by Blount [1936]. Irradiated oxypenicilliopsin is however quite insoluble in sodium bisulphite solution and hence is unlikely to be a derivative of phenanthraquinone.

Comparison of the colour reactions of irradiated oxypenicilliopsin and hypericin

Through the courtesy of Dr G. Mackinney of the Agricultural Experiment Station of the University of California we were kindly provided in December 1938 with a specimen of hypericin, prepared by his colleague Mr Pace from St John's Wort (*Hypericum perforatum*). This specimen had been purified by chromatographic analysis and was almost certainly the purest specimen of hypericin ever prepared up to that time. Prof. Ch. Dhéré and his collaborator, Dr V. Castelli, of the University of Fribourg, compared [Dhéré & Castelli, 1939] the absorption and fluorescence spectra of irradiated oxypenicilliopsin and the above specimen of hypericin, both at ordinary temperatures and at the temperatures of liquid air and liquid nitrogen. The very remarkable spectra given by both substances were found to be indistinguishable from each other.

This evidence, which apparently points to the identity of hypericin and irradiated oxypenicilliopsin is, however, not supported by their colour reactions, carried out side by side with a number of reagents, the results of which are given below. It is evident from these reactions that hypericin and irradiated oxypenicilliopsin are closely related substances but the reaction with alcoholic methylamine is sufficient in itself to prove their non-identity.

Reagent	Hypericin	Irradiated oxypenicilliopsin
Cold conc. H_2SO_4	Green with strong, dull red fluorescence. Stable	Green, with strong dull red fluorescence. Stable
2*N* NaOH	Green. Fades to dirty brown in 10 min. and later gives a flocculent precipitate	Stable green. Green flocculent precipitate overnight
2*N* NH_4OH	Blue with slight green tinge. Becomes brown overnight	Pale mauve. Quite stable
2*N* Na_2CO_3	Slowly dissolves to a blue solution which becomes pale brown overnight	Absolutely insoluble. No colour even overnight
Pyridine	The very dilute solution is definitely purplish in thin layers. Intense red fluorescence	The very dilute solution is brownish-pink in thin layers. Intense red fluorescence
Acetone	Cerise. Intense red fluorescence	Cerise but less blue in tone than with hypericin. Intense red fluorescence
33% wet wt. solution of methylamine in ethanol	Deep green. Becomes brown overnight. No marked fluorescence	Pale violet. Becomes cherry-red overnight and an intense fiery red fluorescence develops

SUMMARY

The colouring matter present in laboratory cultures, grown in the dark at 24°, of the fungus *Penicilliopsis clavariaeformis* Solms-Laubach has been isolated in good yield and has been named *penicilliopsin*. Penicilliopsin, $C_{30}H_{24}O_8$, orange prisms M.P. 330° (decomp.), is oxidized in air in suitable solvents to *oxypenicilliopsin*, $C_{30}H_{20}O_9$, purple-black crystalline aggregates, M.P. above 360°, solutions of which, on exposure to light, are converted into the isomeric *irradiated oxypenicilliopsin*, $C_{30}H_{20}O_9$, chocolate-brown needles M.P. above 370°, which give intensely fluorescent solutions. Reasons are given for the belief that the colouring matter from naturally occurring specimens of *P. clavariaeformis*, which was formerly known as mycoporphyrin, was a mixture of penicilliopsin, oxypenicilliopsin and irradiated oxypenicilliopsin. It is shown that irradiated oxypenicilliopsin is very closely related to, but is not identical with, hypericin the colouring matter of St John's Wort, *Hypericum perforatum*. A number of derivatives and breakdown products of penicilliopsin are described, and since this substance gives *Frangula*-emodin anthranol on heating alone and tetranitroemodin on oxidation with HNO_3 the view is tentatively advanced that penicilliopsin may be a polyhydroxy derivative of a reduced *meso*dimethyldianthrone.

We desire to thank Prof. Ch. Dhéré for much friendly correspondence on the spectra of penicilliopsin, oxypenicilliopsin and irradiated oxypenicilliopsin; Dr G. Mackinney for a gift of hypericin; and our colleagues Mr G. Smith and Dr C. T. Calam for valued assistance during certain parts of the work.

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96. STEROL METABOLISM OF MICRO-ORGANISMS

1. YEAST

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(*Received 4 April 1940*)

The production of sterols, more particularly ergosterol, by micro-organisms is now a well-established fact. It is, especially, a metabolic accomplishment characteristic of the moulds and yeasts; whether bacteria produce sterols is a controversial point, with the weight of the evidence against this supposition. We became interested in elucidating the factors and metabolic intermediates concerned in the synthesis of sterols; and, consequently, in the function of sterols. We chose to use micro-organisms for this purpose, both on account of the uniformity of this biological material and its simple organization. In neither of these respects have micro-organisms entirely come up to our expectations. We give here the results with yeast; other results using other organisms, chiefly *Aspergillus niger*, will form the subject of a separate communication.

Experimental

Cultivation. We adopted a method similar in principle to that used by Smedley-MacLean [1922] in investigating the fat and carbohydrate metabolism of yeast, a method which has the combined advantages of speed and reproducibility. Four lots of moist baker's yeast, each of 20 g., were taken for each experiment. One lot was worked up without further treatment; this is the control. Each of the other three was suspended in 500 ml. of tap water, containing 50 g. of cane sugar and 0·5 g. KH_2PO_4. One lot was aerated; another was allowed to ferment without aeration (i.e. it was essentially anaerobic); and to the last lot, which was aerated, was added the substrate under investigation. Incubation, at 28°, was usually for 3 days, although in some later experiments 1 day was deemed sufficient. Aeration was achieved by making use of the special type of flask (of 2 l. capacity) described by Kluyver *et al.* [1925], in which the incoming air-stream is broken up by means of a sintered glass filter.

Harvesting. The yeast from each flask was harvested by centrifuging, washed once with distilled water and re-centrifuged: the centrifugate was shaken up with 100 ml. of 75% methyl alcohol and allowed to stand at 10° overnight. It was then filtered by suction and the filter cake broken up and air-dried at 32° for 24 hr. The yeast was finally dried over $CaCl_2$ in an evacuated desiccator at 32°, and then ground to a fine powder in a coffee mill.

2 g. of the powder were extracted with a boiling mixture of alcohol (two parts) and benzene (one part), as described by Bills *et al.* [1930]. The filtered extract was evaporated to dryness on the water bath, under reduced pressure, and the residue taken up in 100 ml. of absolute alcohol. Aliquots of this solution were then taken for the estimation of total alcohol-benzene-soluble material

(henceforward designated "total lipin"), the estimation of unsaponifiable matter and of sterol.

Estimations. Unsaponifiable matter was determined by adding 1 ml. of 20% KOH to 20 ml. of the alcoholic solution and taking the solution to dryness on a boiling water bath. The residue, after drying over $CaCl_2$ in a desiccator, was extracted three times with ether. The ethereal solution was filtered into a tared beaker and allowed to evaporate spontaneously.

Sterol was estimated spectrographically, as a general rule, by measuring the extinction coefficient at 280 mμ. The absorption of the alcoholic solution, suitably diluted, was measured with a small Hilger quartz spectrograph, used in conjunction with a rotating logarithmic sector and a Dobson microphotometer. The values obtained were frequently checked by gravimetric estimations on the same solution. This was considered desirable in case there should be any appreciable amount of sterol other than ergosterol, since the spectrographic method only estimates the latter. For instance α-dihydroergosterol would escape measurement by the spectrographic method. Further, the gravimetric method enables one to distinguish between ester and free sterol. Nevertheless, it has its drawbacks; the instability of ergosterol makes it susceptible to the rather drastic treatment involved in digitonide estimations. Further, the solubility of ergosterol digitonide has apparently never been investigated. Most workers have either assumed that it is the same as that of cholesterol digitonide or have ignored the question. In our hands the digitonide method gave excellent results with cholesterol, reasonably good results with solutions of pure ergosterol, and only approximate results with alcoholic extracts of yeast or *Aspergillus* "lipin".

The digitonide was prepared by treating a 20 ml. aliquot of the alcoholic solution with the requisite amount [Dam, 1928] of 1% digitonin in 90% alcohol. The mixture was brought to the boil and taken to dryness on the water-bath. The residue was washed with ether into a tared Gooch filter and there washed with water until frothing ceased. The filter was dried to constant weight in an evacuated desiccator (ergosterol digitonide decomposes when dried at high temperatures, e.g. 105°).

Results

Table 1 brings out several points. First, it is clear, from a comparison of Exps. 1–4 with 5–8, that sterol production is a feature of aerobiosis. It is also clear that the increase in sterol under aerobic conditions is far in excess of the increased growth—a point emphasized later in the composite Table 9. On the other hand the apparent decrease in sterol in Exp. 5 is probably a dilution effect, the ergosterol of the total yeast (6·66 g. in this case) is the same, or slightly greater than, that of the control (4·44 g.). Nevertheless, we record later (see Exp. 15) a real case of decrease in sterol. The second point is concerned with the time of incubation. While increase in the incubation period leads to an increase in ergosterol production in the aerated suspensions, the ergosterol production after only 24 hr. is considerable. In most of our work on the effect of added substrates we have incubated for 72 hr., chiefly for reasons of our own convenience. In some later experiments, however, we cut the time down to 24 hr. The third point is that Table 1 brings out very clearly that most, if not all, of the ergosterol in the aerated yeast is esterified. The discrepancies between the amounts of ergosterol determined spectrographically and by the digitonide method are probably due to other digitonin-precipitable substances in yeast (e.g. α-dihydroergosterol).

Table 1

			4	5	6	7	8
			2 g. of dry yeast contain				
						Sterol by digitonide	
1 Exp.	2 Duration of incubation hr.	3 Yield of dry yeast g.	Total lipin mg.	Unsapon. matter mg.	Ergosterol (spectr.) mg.	Before sapon. mg.	After sapon. mg.
0	Control	4·443	30	4·0	5·3	—	—
				Aerated			
1	24	8·83	123	35·5	18·9	2·6	23·2
2	48	8·92	97	34·5	16·7	2·1	23·0
3	96	9·9	136·5	43·0	25·7	8·3	33·6
4	120	9·47	142·5	44·0	25·0	2·4	29·4
				Not aerated			
5	24	6·66	19·0	6·0	3·5	—	—
6	48	6·48	25·0	5·5	4·2	—	—
7	96	6·01	22·5	7·0	4·5	—	—
8	120	5·7	25·5	5·5	5·3	—	—

Column 4: total lipin represents the material soluble in alcohol-benzene and subsequently in absolute alcohol.

Column 6: ergosterol determined spectrographically.

Columns 7 and 8: sterol determined by digitonin precipitation. (Not attempted in Exps. 5–8.)

In Table 2 we record the results of investigating the effect of sodium acetate upon sterol production, and incidentally the effect of aeration in the absence of any carbon compound (Exp. 10). Smedley-MacLean [1922] has found sodium acetate effective in raising the fat content of yeast; and our own experiments with *Aspergillus niger* led us to believe that sodium acetate was worth investigation as one of the simpler precursors of ergosterol. The results justify this view.

Table 2

			2 g. of dry yeast contain		
Exp.	Conditions of exp.	Yield of dry yeast g.	Total lipin mg.	Unsapon. matter mg.	Ergosterol mg.
9	Control	4·516	27·5	4·0	4·3
10	No carbon	4·495	26·5	27·5	6·3
11	5 g. Na acetate	4·806	35·0	13·5	7·1
12	Cane sugar	8·411	111·5	37·5	20·1
13	Cane sugar + 5 g. Na acetate	8·569	127·0	38·5	24·1

In Exps. 10–13 the common medium was 500 ml. of tap water plus 0·5 g. KH_2PO_4. In Exp. 10 nothing else was added and in Exps. 11, 12 and 13 the nutrients as indicated. The amount of cane sugar was 50 g. Incubation was for 24 hr. with aeration. Ergosterol was determined spectrographically.

We conclude that sodium acetate added to the basal medium leads to a slight increase of ergosterol production. The significance of this is referred to in the discussion.

Many of the commoner possible intermediates in carbohydrate metabolism can be ruled out after a consideration of the results obtained in studying the fat metabolism of yeast, as recorded by Smedley-MacLean [1922] and MacLeod & Smedley-MacLean [1938]. We refer here, of course, to the figures given by them

for unsaponifiable matter. We therefore turned our attention to certain substances as yet untried and, for various reasons, we elected to try in the first place the effect of ascorbic acid. The results are given below.

Table 3

			2 g. of dry yeast contain				
						Sterol by digitonide	
Exp.	Conditions of exp.	Yield of dry yeast g.	Total lipin mg.	Unsapon. matter mg.	Ergosterol (spectr.) mg.	Before sapon. mg.	After sapon. mg.
14	Control	4·671	28·0	18·0	6·8	6·3	7·3
15	Cane sugar anaerobic	6·146	30·0	16·0	3·1	3·2	4·4
16	Cane sugar aerated	8·217	113·0	45·0	21·8	7·3	37·1
17	Cane sugar aerated + ascorbic acid	9·723	138·0	55·0	28·7	8·5	72·0

Incubation was for 72 hr. Amounts of cane sugar and phosphate as in the preceding experiments. The amount of ascorbic acid added in Exp. 17 was 1 g., neutralized with *N* NaOH.

It will be seen that there is a marked increase in the ergosterol content of yeast incubated with aeration in the presence of ascorbic acid. Once again the importance of aeration is demonstrated; and also the fact that most of the ergosterol formed during aeration is esterified. Nevertheless this result is put forward with some reserve because up to the present we have not been able to repeat it. If, for instance, ascorbic acid were exercising any beneficial effect, it would be expected that increasing the amount of the acid would have correspondingly greater effects. Table 4 contains the results of our attempt to test this point.

Table 4

			2 g. of dry yeast contain	
Exp.	Conditions of exp.	Yield of dry yeast g.	Total lipin mg.	Ergosterol mg.
18	Control	4·712	19·0	3·8
19	Cane sugar	9·346	56·0	7·7
20	Cane sugar + 1 g. ascorbic acid	9·138	62·5	8·4
21	Cane sugar + 2 g. ascorbic acid	8·757	60·0	8·1
22	Cane sugar + 3 g. ascorbic acid	9·533	73·5	9·6
23	No cane sugar and no ascorbic acid	4·683	20·0	4·6
24	1 g. ascorbic acid	4·664	19·5	4·6

All incubated at 28° with aeration in the basal medium, tap water and phosphate (except 18).

The above series was, inadvertently, run for only 24 hr.; the original ascorbic acid experiment was run for 72 hr. This may be the reason for the failure to repeat. The matter is still under investigation. Another possibility is variation in the yeast, a point always to be borne in mind when comparing one series of experiments with another. In the work recorded in this paper 7 different batches of yeast were used. The synthetic capacities of 5 of them, as measured by the amounts of ergosterol formed from cane sugar alone, were remarkably similar. The other 2 (one of which was used in Exps. 18–24) were able to form only half the amount of ergosterol formed by the first 5. Table 5 illustrates this.

One of the reasons for trying ascorbic acid was its marked reactivity. The idea that the hexose unit undergoing metabolic change is in an especially "reactive" state is fairly widespread. This view is expressed by Harden [1932], and Massengale *et al.* [1931] resort to the same conception in discussing the conversion of carbohydrate into sterol; von Euler & Nilsson's [1930]

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Table 5. *Ergosterol (in mg.) in 2 g. of dry yeast, after aeration in cane sugar phosphate medium*

Duration days	Different yeasts no.						
	1	2	3	4	5	6	7
1	18·9	20·1	—	—	—	7·7	9·4
2	16·7	—	—	—	—	—	—
3	—	—	21·8	22·1	19·4	—	—
4	25·7	—	—	—	—	—	—
5	25·0	—	—	—	—	—	—

"*am*-hexose" is another version of the same idea. For this reason we thought it worth while to test yeast glycogen as a "baustein" in the synthesis of sterol. The yeast glycogen which we used was hydrolysed immediately before use, with the aid of ptyalin; no attempt was made to separate the reducing sugar so formed; and as a control we used an equivalent amount of maltose. The results are given in Table 6 and provide no encouragement in the attempt to supply "active" carbohydrate. In arriving at this conclusion we are only considering Exps. 25, 26 and 27; these should not be compared with Exp. 28, in which the amount of cane sugar was the usual 50 g.; in the case of Exps. 26 and 27 the amount of sugar was only 2 g.

In Table 6 are two other results, representing two quite different points, but included here because performed on the same batch of yeast. Exps. 28 and 29 are controls. In Exp. 30 the air used for aeration was drawn through ethyl alcohol. Smedley-MacLean & Hoffert [1923] have shown that the addition of ethyl alcohol to the water in which yeast is oxygenated leads to a deposition of fat in the yeast; whilst Sobotka *et al.* [1935] have demonstrated an increase in the fat and sterol content of yeast left in contact with air saturated with alcohol vapour. There was a slight increase in sterol (see Table 6), which was a little more marked in a repetition experiment (not given in the table). But in neither case is there warrant for considering ethyl alcohol as a significant contributor to sterol synthesis. More interesting is the fact which emerges that ethyl alcohol is capable of sustaining growth of yeast aerobically, the actual yield being significantly greater than in the control sample.

Exp. 31 represents the effect of N_2 in place of air. The failure of yeast incubated without aeration to synthesize sterol might be attributed to inadequate nutrition of the yeast. In the non-aerated experiments the yeast settles to the bottom of the flask; consequently it must depend on diffusion for its supply of cane sugar and the lower layers of the settled yeast may be partially starved. In the aerated experiments, on the other hand, the yeast is kept uniformly suspended in the medium, whereby an adequate nourishment is ensured. The accumulation of CO_2 in the non-aerated experiments might also have an adverse effect on sterol synthesis. The substitution of N_2 for air enabled us to test these points; and it will be seen from Table 6 that sterol production in the presence of N_2 is no greater than that obtained in non-aerated experiments. There appears to be an actual decrease, but we are inclined to consider this a dilution effect. It will be noted, however, that the yield of yeast is greater than the yields from non-aerated experiments (cf. Exps. 5, 6, 7, 8 and 15). This probably means that the yeasts in the non-aerated experiments are indeed under-nourished. These experiments offer further proof that sterol synthesis is not dependent on growth; for a growth equal to that attained by the ergosterol-rich, aerated yeast is achieved here without the production of sterol. Exp. 31 has been repeated since with precisely similar results.

Table 6

Exp.	Conditions of exp.	Yield of dry yeast g.	2 g. of dry yeast contain: Total lipin mg.	2 g. of dry yeast contain: Ergosterol (spectr.) mg.
25	Control	4·807	18·5	4·2
26	Maltose	5·457	26·0	6·6
27	Hydrolysed glycogen	4·686	24·0	6·9
28	Cane sugar	9·286	53·5	9·4
29	No carbon compound	4·985	26·5	6·2
30	Alcohol	5·682	26·0	5·7
31	Nitrogen	8·172	17·0	2·5

Incubated for 24 hr. with aeration (except 25 and 31).

The negative character of our findings so far led us to reconsider the possible intermediates. We had hitherto avoided introducing any N compounds, in order to keep as clear as possible of the metabolic processes accompanying growth. Yet the fact that the higher alcohols produced during fermentation are the result of decarboxylic deamination of amino-acids made it imperative that the effect of these should be tested, especially in conjunction with actively fermenting carbohydrate. In the first instance it was considered desirable to use a mixture of amino-acids. Table 7 contains the results of these experiments. Exp. 32 is the usual control; Exp. 33 represents cane sugar without any amino-acids; Exp. 34 cane sugar + a mixture of amino-acids (casein hydrolysed with trypsin) in an amount equivalent to 0·22 g. of amino-N. Exp. 35 is similar to 34 but anaerobic.

Table 7

Exp.	Conditions of exp.	Yield of dry yeast g.	2 g. of dry yeast contain: Total lipin mg.	2 g. of dry yeast contain: Unsapon. matter mg.	2 g. of dry yeast contain: Ergosterol (spectr.) mg.
32	Control	4·333	25·5	12·5	5·4
33	Cane sugar only	8·003	113·5	55·5	22·1
34	Cane sugar + amino-acids	10·984	58·5	26·5	12·6
35	Cane sugar + amino-acids: anaerobic	6·433	22·0	15·5	3·9

Incubation for 72 hr.; Exps. 33 and 34 aerated.

It would thus seem that the addition of amino-acids not only failed to increase sterol production but actually appeared to inhibit it. Despite this result we carried out further experiments, acting on a suggestion made to us by Prof. Robinson, that we should try specifically the effect of tyrosine. The results are contained in Table 8. Exps. 36 and 37 are the usual controls; Exp. 38 is the same as 37, but with the addition of 1 g. of *l*-tyrosine, whilst Exp. 39 is similar to 38 but anaerobic.

Table 8

Exp.	Conditions of exp.	Yield of dry yeast g.	2 g. of dry yeast contain: Total lipin mg.	2 g. of dry yeast contain: Unsapon. matter mg.	2 g. of dry yeast contain: Ergosterol (spectr.) mg.
36	Control	4·543	26·0	6·5	6·0
37	Cane sugar only	9·795	127·5	37·5	19·4
38	Cane sugar + tyrosine	9·480	95·5	34·0	14·6
39	Cane sugar + tyrosine: anaerobic	6·257	28·0	7·5	4·4

Incubated for 72 hr.; Exps. 37 and 38 aerated.

Once again it is quite clear that the addition of tyrosine leads to a decrease rather than an increase in sterol production. We may add here that we have also tried the effect of tyrosol in the system and found it to be the same as that of tyrosine.

In the foregoing tables we have expressed the results uniformly in terms of mg. per 2 g. of dry yeast—the amount actually taken for each estimation. In Table 9 we have brought together the data from the preceding tables in a different form. The first and second columns are self-explanatory. The third column contains the total ergosterol in the whole yeast, for any given experiment, less the amount of ergosterol in the control sample for that series. The fourth column represents a method of expressing the relationship of sterol production to growth. If

E_f = ergosterol in total yeast at conclusion of experiment,
E_0 = ergosterol in total yeast at beginning of experiment,
Y_f = total yeast at conclusion of experiment,
Y_0 = total yeast at beginning of experiment,

then the ratio expressed in the third column is $\frac{E_f/E_0}{Y_f/Y_0}$.

From this it follows that if sterol production ran parallel with growth, the value obtained should be 1.

Table 9

Conditions of exp.	Ergosterol % of dry yeast	Net increase in ergosterol mg.	Ratio sterol/growth
Control	0·26	—	—
	Aerobic		
Phosphate	0·29	3·9	1·38
Phosphate + acetate	0·36	7·4	1·66
Phosphate + ascorbic acid	0·27	3·9	1·35
Phosphate + EtOH	0·30	7·9	1·47
Cane sugar (Cs.)	1·06	82·6	3·87
Cs. + ascorbic acid	1·44	123·6	4·22
Cs. + acetate	1·21	93·6	5·61
Cs. + amino-acids	0·63	57·5	2·34
Cs. + tyrosine	0·73	55·6	2·44
Cs. + tyrosol	0·70	48·7	3·25
	Anaerobic		
Cs. + nitrogen	0·14	1·6	0·68
Cs.	0·21	0	0·75
Cs. + amino-acids	0·20	0·8	0·72
Cs. + tyrosine	0·22	0·2	0·73

It should be understood that phosphate was present in all experiments, in addition to the substances indicated. The figures for the ascorbic acid experiment given above are for the 3-day run; those of the 1-day run are not included here as the particular yeast used was abnormal in other respects (see Table 5). The relevant figures for that particular yeast (Table 4) showed that there was no effect due to ascorbic acid.

Discussion

There are many different views concerning the mechanism of formation of sterols, a subject intimately bound up with the question of the metabolic function of sterols. Knowledge of the one is almost bound to illuminate the other. Perhaps the most attractive suggestion concerning sterol formation is that it arises by cyclization of a carotenoid or polyene chain. Thus Robinson [1934] considered squalene a possible precursor, whilst Bryant [1935] put

forward the merits of carotene for the role. Others [e.g. Artom, 1923] have postulated cyclization of oleic acid. Massengale *et al.* [1931] investigated the effectiveness of various sugars in the production of sterols by yeast and Bills [1935], reviewing the subject, infers that sterols are mainly the products of carbohydrate metabolism. Smedley-MacLean & Hoffert [1926] in a paper primarily concerned with fat metabolism of yeast, record the unsaponifiable matter found under the influence of a variety of substrates; this contributes valuable information concerning possible intermediates, but no positive suggestion arises from it. Nor can we, on the basis of the work reported here, point to any specific intermediate as the precursor of sterol. Our findings can be summarized under two headings. (1) We are able to exclude a certain number of substances as precursors, with a fair degree of confidence; and (2) we have extracted some information concerning the general metabolism of sterols by yeast.

Dealing with the first point, the evidence that amino-acids do not play any part in sterol formation seems conclusive. This is in agreement with the findings of previous workers [Massengale *et al.* 1931; Reindel *et al.* 1937; and Sobotka *et al.* 1935], who could demonstrate no connexion between sterol synthesis and N metabolism. (We can offer no explanation for the rather surprising discovery that the amino-acid mixture, tyrosine and tyrosol, all inhibited sterol production.) Nor can we find any support for the view that a "reactive hexose" is concerned in sterol formation; the results with hydrolysed yeast glycogen are negative. The effect of ascorbic acid is uncertain and that of ethyl alcohol has already been discussed.

We believe, on the basis of our experiments, that sodium acetate can provide the necessary carbon for sterol synthesis. It is known that acetate stimulates the formation of fat in yeast [Smedley-MacLean & Hoffert, 1923], and more recently Sonderhoff & Thomas [1937] have shown that the unsaponifiable material of yeast grown in a solution of trideutero-acetate contains 31·0 atoms % of deuterium. It is interesting to note in this connexion that Rittenberg & Schoenheimer [1937] concluded from a similar study, using deuterium as an indicator, that the synthesis of cholesterol in the animal body involves the coupling of a large number of small molecules. Further support for the view that acetate can serve as a sterol precursor may be found in the fact that *Aspergillus niger* can synthesize ergosterol when sodium acetate is its sole source of carbon [Maguigan, unpublished].

Under the second heading we have established five points.

(*a*) Sterol formation occurs at an early stage in any fermentation where it occurs at all. We may infer from this that it is a synthetic and not a degradative feature.

(*b*) Most of the sterol formed is present as ester. This has been claimed before [Smedley-MacLean, & Thomas 1920] and our experiments consolidate the point. If sterol formation be considered a consequence of the increased fat formation encountered under aerobic conditions, then an idea worth considering is that the sterol here is acting as a detoxicating agent against the excessive production of fatty acids; "neutralizing", as it were, by their esterification those fatty acids destined for fat storage in contradistinction to esterification with glycerol (or choline glycerophosphoric acid), a catabolic step.

As far as we are aware, the only sterol ester recorded as occurring in micro-organisms is ergosteryl palmitate, isolated from *Penicillium brevi-compactum* by Oxford & Raistrick [1933]. Our efforts to isolate and characterize the sterol ester of yeast fat have thus far been unsuccessful; but we are continuing with this point.

(*c*) The view, set forth above, that sterol formation may be a consequence of increased fat formation would lead one to expect a constant relationship between the sterol content and total lipin of yeast. Our results show that this relationship does tend to be constant, despite fairly wide variations in individual estimations. The average value (45 experiments) of the ergosterol content expressed as percentage of total lipin is 18·3 %. In this conclusion we agree with Sobotka *et al.* [1935] and disagree with Bills [Massengale *et al.* 1931] who states that there is no relationship between the total lipin and sterol contents of yeast.

(*d*) Sterol formation is not directly related to growth. Not only is the rate of sterol formation much greater than the rate of growth, under the most favourable conditions investigated, but the former does not always vary in the same direction as the latter. Even in those experiments in which there was no growth, a small but definite increase in sterol was noted. It is true that a considerable growth occurred in the experiments yielding the largest amounts of ergosterol, but in these cases the greater sterol yields were often accompanied by lesser growths, and *vice versa*. In this connexion, it may be noted that the average value of the ratio: final weight of yeast/original weight of yeast for a group of 20 experiments in which the yeast was aerated in the presence of cane sugar with and without the addition of simple organic compounds was 2·00. The maximum variation in this ratio was $\pm 12\,\%$, showing that with respect to growth under the given conditions the yeast behaved as uniformly as can be expected of a biological population. The variations in sterol synthesis in the same experiments were, on the other hand, very great indeed.

The findings of Bills *et al.* [1930] and Reindel *et al.* [1937] that sterol synthesis tends to run parallel with growth can be explained by supposing that, in general, conditions favourable to growth are also favourable to sterol production. Under the conditions of our experiments we have been able to dissect the two processes.

(*e*) Sterol formation is a feature of aerobiosis. This is a conclusion which is capable of extension to organisms other than yeast. Without committing ourselves on the subject of sterol function in mammals (where it seems to be multiple), there must be some really fundamental function, involving sterol, common to the physiological processes of animals and plants (including fungi). To suppose that this process is intimately associated with aerobiosis is consonant with all the known relative facts. It is in accord also with the absence of sterols from bacteria where these are anaerobes; controversial cases may be due to the use of facultative anaerobes; aerobes should contain sterols. Evidence on this point is being sought.

Summary

1. The production of sterols by yeast under a variety of conditions has been studied.

2. Of various substrates examined, sodium acetate led to a slight increase in sterol; ascorbic acid gave a definite increase in one case, and no effect in others; hydrolysed yeast glycogen was not effective; the role of ethyl alcohol as a sterol precursor is open to doubt; a mixture of amino-acids, tyrosine and tyrosol respectively, inhibited sterol formation.

3. It is established that sterol production (*a*) is a feature of aerobic metabolism, (*b*) arises early in the course of fermentation, (*c*) is accompanied by a corresponding increase in total lipin and (*d*) develops at a greater rate than growth, and is not directly correlated with it. (*e*) Most of the new sterol formed is esterified.

The authors acknowledge the interest of Prof. R. A. Peters in this work.

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97. THE EFFECT OF OESTRONE ON THE METABOLISM OF RAT UTERUS[1]

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(*Received 30 April 1940*)

It has been established that injection of oestrogenic hormone causes an increase in the rate of metabolism of certain tissues. This has been shown for uterus by several workers (for literature see Tesauro [1936]), for ovary by Kiyohara & Izawa [1936], for anterior pituitary and for liver by Victor & Andersen [1937], Andersen & Victor [1938]; the latter workers, as well as Kiyohara & Izawa, showed further that kidney tissue is unaffected by injection of hormone. Another effect of injecting such hormones is to produce rapid growth of the uterus. This growth can be measured by observing either the increase in dry weight [Astwood, 1938; Varangot, 1939] or the number of cell divisions after injection of colchicine [Allen *et al.* 1937]. Both methods show that growth can first be observed 6–9 hr. after injection, and that it is maximal after 36 hr. The results to be reported here show that the changes in metabolic rate closely follow these growth changes, the first increase occurring from 6 to 12 hr. after injection, whilst the maximal rate is reached after 24–36 hr. During the subsequent regression period metabolic rate is depressed, the original rate being regained 96 hr. after injection, when the weight has returned to the pre-injection value [Varangot, 1939].

Methods

Rats aged about 4 months were used for experiment from 17 to 43 days after bilateral oöphorectomy. No influence of the time after operation on the results was observed. For two series of experiments younger rats were used, in one oöphorectomy was performed at 6 weeks, hypophysectomy 1 week later and the rats were used after the lapse of a further 5 days; the second series, which served as controls for the hypophysectomized rats, were oöphorectomized only at the age of 6 weeks.

Isolated uteri, in a glucose-Ringer medium, were used in all cases, and the aerobic and anaerobic metabolic rates measured by the methods previously described [Kerly, 1937].

Crystalline oestrone was used for all injections; both it and the oestriol used in some of the *in vitro* experiments were prepared by Prof. Marrian. A stock solution of oestrone in 10% alcohol was prepared and diluted with 10 vol. of water immediately before use; 5 subcutaneous injections of 0·5 ml. (=0·5 μg. oestrone) were given at 0, 6, 22, 26 and 30 hr. [see Voss, 1936]. The activity of all preparations was checked by testing the vaginal smears; cornified cells were observed in some cases 48 hr. after, and in all cases 72 hr. after, the first injection.

Anaerobiosis

The rates of anaerobic glycolysis of uteri from uninjected rats showed great variation (Fig. 1), ranging from the high values typical of uteri from intact rats in pro-oestrus to the low values of di-oestrus. The few values previously

[1] Some of the material contained in this paper was reported at a meeting of the Biochemical Society in March 1938.

reported for oöphorectomized rats [Kerly, 1937] chanced to come from animals showing low values. Zuckerman [1938] has suggested that the accessory reproductive organs of spayed female animals undergo cyclic changes due to the influence of some extra-ovarian factor. This would explain the large variation in metabolic rates of uteri from oöphorectomized rats, where there is no longer any change in the vaginal smear and therefore no means of judging the stage of the uterine cycle.

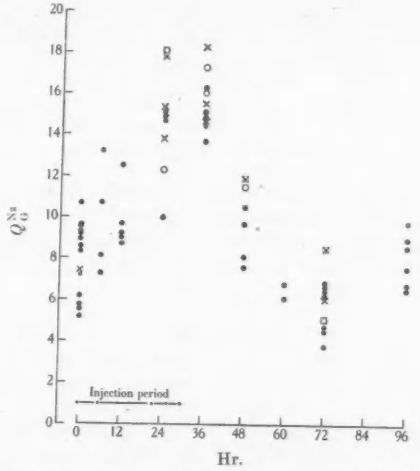

Fig. 1. The effect of injection of oestrone on the anaerobic glycolysis of isolated uterus. • Oöphorectomized rats, aged approximately 4 months. o Oöphorectomized rats, aged approximately 2 months. × Hypophysectomized and oöphorectomized rats, aged approximately 2 months. Values plotted at 0 hr. are those of uteri from uninjected rats.

After injection of oestrone some rise in the rate of anaerobic glycolysis was observed 6 and 12 hr. after the first injection (Fig. 1), but as the rate before injection varied over such a wide range it was difficult to be certain of small increases. After 24 hr. there was a large increase and after 36 hr., when the maximal rate had been reached, the rate was almost double that of uteri from uninjected rats. At this time there was no change in the vaginal smear. After 48 hr., when both cornified and epithelial cells were usually present, the glycolytic rate had fallen; this fall continued until, after 72 hr., the rate was somewhat lower than for uninjected rats. 24 hr. later (96 hr. after the first injection) it had risen again to the original value. Injection of a large single dose of oestrone did not produce an earlier increase in rate; 6 hr. after injection of 5 and 25 μg. oestrone the values found for $Q_G^{N_2}$ were 9·6 and 9·1 respectively. The high rate of anaerobic glycolysis induced by injection of oestrone was much greater than the pro-oestrus values of the normal cycle (mean $Q_G^{N_2}$ 9·7 [Kerly, 1937]), but there was a similar time interval (36 hr.) between the highest and lowest rates observed after injection and the high pro-oestrus and the low late oestrus values (mean $Q_G^{N_2}$ 3·5) found in the intact rat.

If injections were continued beyond the standard 30 hr. period (2 injections daily of 0·5 μg. oestrone) the very high 36 hr. values were not maintained (Table 1). 72 hr. after the initial injection the glycolytic rate had fallen below

Table 1. *Anaerobic glycolysis of isolated uterus after prolonged injection of oestrone*

0·5 μg. oestrone injected at the following times: 0, 6, 22, 26, 30, 46, 54, 70, 78 and 96 hr. Times for $Q_G^{N_2}$ reckoned from the first injection.

$Q_G^{N_2}$ at		
36	72	100 hr.
15·1	9·3	10·4
16·2	8·7	11·0
13·7	—	10·7
14·7	—	—
14·6	—	—

the 36 hr. value, but not to so great an extent as in those rats in which injections ceased at 30 hr.; after 100 hr. the rate had risen again to an intermediate value. The cyclic character of the response to the initial injections appears to be maintained in spite of further injection of hormone. As the time after injection increased there was less variation of rate between uteri from different rats, which would be expected if an artificial cycle had been superimposed on animals initially at different phases of their natural cycles.

Aerobiosis

The rates of aerobic metabolism reported here are considerably higher than those recorded previously both for intact and oöphorectomized rats [Kerly, 1937]. This is due to the fact that an error was made in calculating the earlier values,

Table 2. *Aerobic metabolism of isolated uterus*

	$-Q_{O_2}$	$Q_G^{N_2}$
(*a*) Normal rats:*		
1. Pro-oestrus	9·0	1·2
2*a*. Early oestrus	9·0	2·1
2*b*. Late oestrus	6·4	2·8
3. Met-oestrus	7·8	3·8
4*a*. Early di-oestrus	6·2	4·9
4*b*. Late di-oestrus	7·9	3·5
(*b*) Oöphorectomized rats	3·7*	3·3*
	1·2	1·4
	4·5	1·7
	8·5	3·2
	3·3	2·8
(*c*) Oöphorectomized rats, injected with oestrone:		
24 hr. after injection	3·3	3·3
	5·2	1·5
36 hr. after injection	7·6	4·7
	8·0	2·8
	9·0	2·6
	10·0	5·8
48 hr. after injection	9·2	3·1
72 hr. after injection	6·2	4·0
	6·9	3·6
	7·4	3·9
	6·1	2·4
	5·2	3·5
96 hr. after injection	5·4	3·5
	6·2	3·5
	6·8	5·2

* Mean values, corrected, of results previously reported [Kerly, 1937].

which require to be multiplied by 2. The mean values given in Table 4 of the former paper have been recalculated and the corrected values are given here in Table 2. The conclusions previously reached are not affected and the corrected results for rate of O_2 consumption for uteri from intact rats at different phases in the reproductive cycle are now in good agreement with those of Khayyal & Scott [1934–5].

As in the anaerobic experiments, large variations in rate of both O_2 consumption and aerobic glycolysis were found in uteri from uninjected rats. Increases in O_2 consumption after injection of oestrone (Table 2) were not so striking as the increases in anaerobic glycolysis, but a significant increase was found 36 hr. after the first injection; after 72 hr. the rate had returned to, but had not fallen below, the pre-injection level. The rate of aerobic glycolysis varied so much, and with the small amount of tissue available the individual values were not sufficiently accurate, to be certain of any changes of rate consequent upon injection of oestrone.

Hypophysectomized rats

Victor & Andersen [1937] found that theelin injected into oöphorectomized rats causes a transitory rise in O_2 consumption of the anterior pituitary, lasting less than 12 hr., whilst its effect in increasing the metabolism of the liver is apparent much later, 72 hr. after injection [Andersen & Victor, 1938]. They were able to show [Victor & Andersen, 1938] that the effect on the liver is indirect, due to the action of the thyroid, itself stimulated by the increased activity of the anterior pituitary. In order to determine whether the effect of oestrone on the uterus was also indirect and due to stimulation of the anterior pituitary, in one series of animals the hypophysis was removed as well as the ovaries. Anaerobic glycolysis was measured rather than O_2 consumption, since the effect of injection was so much more pronounced on anaerobic metabolism. Increases in glycolytic rate occurred at the same time intervals after injection as in those rats which had been only oöphorectomized (Fig. 1). The increases observed were rather greater than in the main series of experiments, probably because younger rats were used; a control series of rats of the same age, which had had only their ovaries removed, gave similar values (Fig. 1).

In vitro *action of oestrogenic hormones*

Victor & Andersen [1937] were able to show that theelin has a direct *in vitro* action on the O_2 consumption of anterior pituitary tissue from oöphorectomized rats, but they found that it had no effect on liver slices, results in accord with their demonstration of the secondary effect of theelin in raising the metabolic rate of the liver. No increase in anaerobic glycolysis was observed during a 2–3 hr. experimental period when oestrone or oestriol was added to isolated uteri from either normal or oöphorectomized rats (Table 3). One horn of the uterus was used as a control, and hormone dissolved in various solvents was added to the Ringer solution surrounding the other horn, an equal amount of solvent being added to the control horn. The very high values found in several experiments must be attributed to the action of the solvent and not to that of the hormone. It is possible that uterine tissue is less permeable than anterior pituitary and that in these experiments no hormone penetrated to the active tissue. On the other hand the failure to show an increase in glycolytic rate may be due to the fact that, although oestrone would appear to act directly on the uterus, and not indirectly through its action on the hypophysis, the increased metabolic rate following its injection is correlated with the uterine growth which it induces and which may occur only in the intact animal.

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Table 3. *Effect of adding oestrone or oestriol* in vitro *on anaerobic glycolysis of isolated uterus*

	$Q_G^{N_2}$	
	Control horn	Experimental horn
1. 0·1 μg. oestrone (dissolved in 0·1 ml. 1% alcohol) added to 3 ml. Ringer	5·5	5·2
2. 2·0 μg. oestrone (dissolved in 0·2 ml. 10% alcohol) added to 3 ml. Ringer	14·8	18·3
3. Uterus soaked 6 hr. in oxygenated Ringer containing 2·0 μg. oestrone (dissolved in 0·2 ml. 10% alcohol) transferred to fresh Ringer for measurement of glycolysis	14·4	13·1
4. As no. 3, but oestrone dissolved in sesame oil	16·3	16·1
5. 25 μg. oestrone dissolved in 2*N* NaOH, neutralized with HCl and made to 3 ml. with Ringer (Victor & Andersen's method)	11·0	9·5
6. 25 μg. oestrone dissolved in 2*N* NaOH, neutralized with HCl, diluted with Ringer and placed in the side arm of the flask; total vol. 3 ml. Control = $Q_G^{N_2}$ before addition of oestrone, experimental = $Q_G^{N_2}$ after tipping in hormone, 1 hr. after beginning of anaerobiosis	11·0	8·3
7. As no. 5, but 25 μg. oestriol used instead of oestrone	5·4	3·2

Summary

1. Anaerobic glycolysis of uteri from oöphorectomized rats showed great variation in rate, ranging from the high values typical of pro-oestrus to the low values found in late di-oestrus in intact rats.

2. Following injection of oestrone an increase in the rate of anaerobic glycolysis was observed after 6–12 hr., but the maximal rate was not reached until 24–36 hr. after injection, at the same time as maximal growth is known to occur. After 72 hr. the rate had fallen below, but after 96 hr. it had returned to, the pre-injection level; at this time the weight is known to return to the original value.

3. If injections were continued for 5 days the glycolytic rate was not maintained at the maximal level.

4. The effect of injection on the O_2 consumption was not so pronounced as on the rate of anaerobic glycolysis, but 36 hr. after injection the rate of O_2 consumption had increased.

5. Hypophysectomy did not affect the action of oestrone in raising the rate of anaerobic glycolysis.

6. Neither oestrone nor oestriol had any *in vitro* action on the rate of anaerobic glycolysis of isolated uterus.

It is with pleasure that I thank Dr Noble, to whom I am greatly indebted for performing the hypophysectomies.

The expenses of this research have been defrayed by a grant from the Government Grant Committee of the Royal Society.

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98. TISSUE METABOLISM OF BRAIN CORTEX AND LIVER AFTER HYPOPHYSECTOMY AND TREATMENT WITH THYROTROPHIC HORMONE

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(*Received 18 April 1940*)

The experiments described in this paper were carried out to determine the effect of hypophysectomy and hyperthyroidism (induced by the thyrotrophic hormone of the anterior lobe of the pituitary) on the metabolism of the cerebral cortex measured *in vitro*, and to compare this tissue with another organ, namely liver.

Up to now comparatively little work has been done from this point of view and, so far as brain is concerned, we know only the paper of Cohen & Gerard [1935] who reported that 16–19 days' treatment with thyroid powder resulted in an increase in respiratory activity of rats' brain measured in whole brain brei. As regards liver, it has already been found that injection of thyrotrophic hormone produced an increased O_2 uptake of liver and kidney. Hypophysectomy was shown to reduce the O_2 consumption of liver slices about 27% [Reiss *et al.* 1933, 1, 2]. The latter fact was confirmed by Victor & Andersen [1938].

Methods

Our experiments were carried out on male rats, some normal, some 3–10 weeks after hypophysectomy. The operation was performed by the paratracheal route and its completeness was checked before use of the animals (*a*) by the lack of increase in weight, (*b*) by atrophy of the gonads and (*c*) in some cases by the appearance of the sudanophobic zone in the adrenal cortex of one of the suprarenal glands which had been removed for examination [Reiss *et al.* 1936]. After killing, the sella turcica was examined to ensure that the hypophysis had been fully removed.

The tissue respiration was measured in cerebral cortex and liver slices according to the methods of Warburg. O_2 uptake was determined by the direct method employing Ringer solution according to Warburg. It may be mentioned that a higher respiration rate for brain slices may be obtained in phosphate-Ringer, especially if Ca is omitted altogether, but we prefer to use for the direct method Ringer solution as follows: 96 parts 0·15 *M* NaCl, 2 parts 0·15 *M* KCl, 2 parts 0·15 *M* $CaCl_2$, 2 parts 0·15 *M* $NaHCO_3$, 2 parts 10% glucose.

Normal and hypophysectomized rats were treated with thyrotrophic hormone. The material used was a preparation containing 20 guinea-pig units per mg. and was not purified so far that it would not produce antihormonal reactions, but it was free from gonadotrophic and corticotrophic factors.

Results

Reference to Table 1 shows that in the case of the unoperated animals treated with thyrotrophic hormone, the O_2 consumption of liver begins to show an increase as compared with normal controls on the second day after starting

[1] Working with Medical Research Council Grant.

the treatment, and reaches maximal values 4–6 days after treatment begins. The cerebral cortex of these animals, on the other hand, shows no appreciable effect up to the third day. From the fourth day onwards an increase was observed in some cases, but this effect was not constantly apparent.

Effect of thyrotropic hormone on metabolism of brain and liver slices

Table 1. *Unoperated rats*

Treatment units	Duration days	Brain		Liver	
		Q_{O_2}	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{N_2}$
—	—	− 8·6	19·6	− 13·0	1·3
—	—	− 5·8	22·7	− 11·2	1·1
—	—	− 5·7	17·0	− 12·7	1·5
—	—	− 7·8	21·9	− 11·5	1·9
—	—	− 8·8	19·8	− 13·2	1·6
100	1	− 7·3	21·1	− 11·7	1·8
100	1	− 6·7	21·0	− 11·4	0·7
100	1	− 5·4	18·5	− 12·3	1·8
300	2	− 6·6	17·0	− 14·3	1·2
300	2	− 7·8	21·7	− 13·2	2·2
300	2	—	—	− 12·2	2·4
400	3	− 7·9	16·1	− 13·2	1·8
400	3	− 5·5	—	− 13·2	2·7
400	3	− 7·0	19·4	− 13·0	2·8
400	3	− 7·3	—	− 13·3	4·5
500	4	− 10·2	21·0	− 14·4	2·2
500	4	− 8·7	19·5	− 15·1	3·0
500	4	− 9·2	17·4	− 14·0	1·7
600	4	− 9·1	23·0	− 23·0	5·4
700	5	− 13·2	20·2	− 11·6	2·0
800	6	− 7·5	23·2	− 15·1	2·3
800	6	− 9·8	25·3	− 23·0	3·1
800	6	− 8·2	—	− 17·4	1·3

Table 2. *Hypophysectomized rats*

Treatment units	Duration days	Brain		Liver	
		Q_{O_2}	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{N_2}$
—	—	− 7·2	25·7	—	3·0
—	—	− 7·9	25·5	− 9·0	5·2
—	—	− 7·1	23·9	− 6·6	3·1
—	—	− 9·4	27·6	− 9·3	2·5
—	—	− 7·5	26·3	− 9·9	3·7
250	5	− 16·9	23·3	− 16·0	3·0
500	5	− 8·5	25·1	—	1·4
500	5	− 7·2	22·7	− 13·3	—
600	6	− 8·9	14·4	− 10·8	1·2
600	6	− 11·3	—	—	—
800	8	− 10·2	—	—	—
800	8	− 11·2	14·9	− 11·8	2·3
2400	16	− 6·4	25·9	− 7·8	2·2
2400	16	− 7·2	19·1	− 11·8	2·8
2400	16	− 6·5	29·2	− 12·1	2·0

After hypophysectomy (Table 2) the O_2 consumption of liver shows a distinct diminution as compared with normal controls, while that of brain is not affected. The anaerobic glycolysis of brain appears to be somewhat (*ca.* 25 %) increased after hypophysectomy. When the hypophysectomized animals were treated with thyrotrophic hormone, the O_2 uptake not only of the liver but of the

cerebral cortex was increased. This latter effect occurs more regularly than in the case of the unoperated animals already mentioned.

The anaerobic glycolysis of brain which showed an increase after hypophysectomy began to decrease after 5–8 days' treatment with thyrotrophic hormone. After 16 days' treatment the effect on brain cortex was no longer evident.

Discussion

It can easily be understood why the O_2 consumption of brain in intact animals should be less subject to endocrinological influence than the liver, the former organ being much more constant in its function than the latter. Also the greater ease with which, in the hypophysectomized animal, the O_2 consumption of the brain could be increased by treatment with thyrotrophic hormone might find an explanation in the fact that, after hypophysectomy, the system of inter-regulation of the various ductless glands is disturbed; whereas in the normal animal attempts to increase the function of one gland will bring compensatory mechanisms into play. It has already been shown that the hypophysectomized animal is much more thyroxine-sensitive than the normal [Reiss & Fischer-Popper, 1936].

The fact that the O_2 consumption of the brain cortex is decreased again after 16 days' treatment with thyrotrophic hormone can perhaps best be explained on the assumption that the so-called "antihormones" are being developed.

As regards the increased rate of anaerobic glycolysis of cerebral cortex slices following hypophysectomy, we are not yet in a position to give an explanation.

Summary

After hypophysectomy there is no significant decrease of O_2 consumption by brain slices (contrary to the behaviour of liver slices of the same animal) but an increase in anaerobic glycolysis of about 25%.

After treatment of hypophysectomized rats with thyrotrophic hormone, the O_2 consumption of brain was found to be increased, as compared with normal unoperated animals, between 5–8 days after commencement of treatment. The liver O_2 uptake was restored or slightly increased above the normal. After 16 days' treatment, the effect on brain was no longer apparent, and that on liver less marked, due presumably to the development of the so-called "antihormones".

After treatment of normal unoperated animals with thyrotrophic hormone, the O_2 consumption of brain was much less markedly increased than in the case of hypophysectomized animals.

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99. THE QUANTITATIVE ESTIMATION OF GLYCURONIC ACID AND ITS CONJUGATED COMPOUNDS BY MEANS OF THE NAPHTHORESORCINOL TEST OF TOLLENS[1]

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Glycuronic acid and its conjugated compounds represent a group of substances about the fate of which in the animal body there is comparatively little information. So far we lack even experimental data which would enable us to decide whether the conjugation of the various substances with glycuronic acid is merely an expression of the detoxicating function of the animal organism or whether it represents a preliminary stage in the metabolism of cyclic compounds. The reason for this slow progress in the field of the metabolism of glycuronic acid and its derivatives is closely connected with technical difficulties involved in their quantitative estimation. The methods now in use are based on the reducing properties of these compounds, on the formation of furfuraldehyde and CO_2 on heating with acids, and on optical rotation measurements. None of these methods is specific and therefore they cannot be regarded as really suitable for quantitative biochemical estimation. The most specific qualitative test is the naphthoresorcinol test of B. Tollens [1908], which depends on the formation of an ether-extractable coloured compound when glycuronic acid or its derivatives are heated with naphthoresorcinol and conc. HCl. C. Tollens [1909] made attempts to adapt this qualitative test for the quantitative analysis of glycuronic acid in urine. Later Neuberg and his co-workers [Mandel & Neuberg, 1908; Neuberg, 1910; Neuberg & Saneyoshi, 1911; Neuberg & Kobel, 1931] have studied the specificity of the test and the effect upon it of various substances. Neuberg found that the presence of several substances met with in biological fluids greatly influences the intensity of the test. He found also that the naphthoresorcinol test is specific for acids containing a carbonyl group and that its sensitivity is enhanced if glycurone be first converted into an osazone. Furthermore, he recommended the use of benzene instead of ether and also less concentrated HCl. He came to the conclusion that the Tollens test cannot be adapted for quantitative purposes. Nevertheless, this comparatively simple test has been used from time to time, and more recently Salt [1935] found that the intensity of colour in the test is more or less proportional to the concentration of glycuronic acid; he was however unable to work out the conditions for reproducible results. Fürth & Peschek [1936] endeavoured to adapt the test for quantitative estimation, but they were discouraged by what they called "the capriciousness" of the reaction. Other authors were more successful; Florkin [1937] obtained fairly good results with solutions of pure glycurone and its salts. He purified the precipitate obtained on heating with naphthoresorcinol and after drying dissolved it in benzene and estimated the colour in the Pulfrich photometer. He gives however

[1] A preliminary account was given by Mozołowski & Rogowski [1939] and Mozołowski *et al.* [1939].

no data for estimation in blood or urine. Lipschitz & Bueding [1939], in their interesting paper on the biological mechanism of glycuronic acid conjugation, also use the naphthoresorcinol test for the estimation of glycuronic acid. They first remove from the alkalinized solution all ether-soluble substances and then re-extract with ether after acidification. After the removal of ether by evaporation they perform the test on the residue. They compare the colour obtained after heating with naphthoresorcinol in strong HCl with that given by standard solutions containing 0·005–0·1 mg. of glycurone. Control estimations with solutions of pure *l*-menthylglycuronic acid, after the addition of borneol, glucose and borneol glucoside, appear to have given satisfactory results. Maughan *et al.* [1938] have described a method of estimation using an aqueous instead of the usual alcoholic solution of naphthoresorcinol and peroxide-free ether. They have applied this modified method to the estimation of pure solutions of the Na salt of pregnandiolglycuronic acid and also to urine to which known amounts of this compound were added.

As can be seen from this brief survey of the available literature it appears that while some authors had poor results others claim to have been able to use Tollens' test for quantitative purposes.

Experimental data are given below which represent results obtained with the modification of Maughan *et al.* [1938] applied to other derivatives of glycuronic acid. Attempts to adapt this method to the estimation of glycuronic acid and its derivatives in blood and urine are also described and the contradictory statements of the authors quoted as to the suitability of the naphthoresorcinol test for quantitative measurements are explained.

Method

The estimations were carried out according to Maughan *et al.* [1938]. The solution of naphthoresorcinol was always prepared on the day before the experiment by dissolving 0·2 g. of naphthoresorcinol (Merck) in 100 ml. water and leaving overnight at 37°. The solution was filtered and used only on that day. The solution to be examined was measured into test tubes and if necessary made up to 2 ml., then 2 ml. naphthoresorcinol were added and, from a burette, 2 ml. conc. HCl. After mixing the contents, the tubes were heated for 30 min. in a boiling water bath and afterwards cooled in ice water for 10 min. To each sample 2 ml. of ethyl alcohol were added and the whole sample was transferred into a 100 ml. glass-stoppered measuring cylinder, the test tubes being washed out 3 times with 5 ml. pure ether. Next the cylinders were shaken fairly rapidly about 10 times in 30 sec. When the ether layer separated, its absorption was determined in the Pulfrich photometer in a 20 mm. cell using the S 57 filter against a control solution prepared by treating distilled water in exactly the same manner.

The estimation of glycuronic acid in pure solutions of its derivatives

Maughan *et al.* [1938] state that their modification of the Tollens test gives good results on solutions of pure Na pregnandiolglycuronate. Experiments given below show that it can also be applied satisfactorily to solutions of glycurone, glycuronic, benzoylglycuronic and borneolglycuronic acids. The following aqueous solutions were used: 4% glycuronic acid ($[\alpha]_D^{20^\circ} = +18^\circ$ immediately after dissolving and 1 hr. later $[\alpha]_D^{20^\circ} = +35^\circ$); 5% glycurone ($[\alpha]_D^{20^\circ} = +18^\circ$); bornylglycuronic acid made according to Quick [1927] and Goebel & Babers [1933], 0·5 g. titrated to phenolphthalein required 14·5 ml. *N*/10 NaOH; 2·5% benzoylglycuronic acid prepared according to Quick [1926] ($[\alpha]_D^{20^\circ} = -25{\cdot}2^\circ$).

The determinations were carried out on solutions of these substances containing equivalent amounts of glycuronic acid, taking mol. wt. 194 for glycuronic acid, 176 for glycurone, 348 for bornylglycuronic and 298 for benzoylglycuronic acid.

Table 1. *Extinction coefficient of ether extracts from the naphthoresorcinol test in solutions of glycuronic acid and its derivatives*

Equivalent of glycuronic acid taken (mg.)	Glycuronic acid	Glycurone	Benzoylglycuronic acid	Bornylglycuronic acid
0·0125	0·12, 0·10	0·12, 0·12	0·12, 0·10	0·11, 0·11
0·025	0·22, 0·22	0·22, 0·22	0·24, 0·24	0·20, 0·22
0·0375	0·36, 0·33	0·35, 0·34	0·34, 0·35	0·31, 0·33
0·050	0·44, —	0·44, 0·43	0·44, 0·43	0·41, 0·43

From the data in Table 1 it can be seen that the reading values are proportional to the concentration of glycuronic acid within the range 0·0125–0·05 mg. glycuronic acid.

When however the amount is increased above 0·05 mg. a precipitate is formed on heating with naphthoresorcinol, and the photometer reading of the ether extract no longer corresponds to the amount of glycuronic acid. Readings for 0·10 mg. glycuronic acid are extremely variable (extinction coefficient values actually found were 0·44, 0·42, 0·31, 0·28, 0·78); even for 0·06 mg. the values obtained are too small and not readily reproducible (Table 2).

Table 2. *Extinction coefficient of ether extracts from the naphthoresorcinol test in solutions of glycuronic acid and its derivatives*

Equivalent of glycuronic acid taken (mg.)	Glycuronic acid	Glycurone	Benzoylglycuronic acid	Bornylglycuronic acid
0·01	0·09	0·09, 0·09	0·10	0·09
0·02	0·18	0·18, 0·20	0·19	0·18
0·03	0·25	0·27, 0·28	0·27	0·27
0·04	0·34	0·35, 0·37	0·38	0·36
0·05	0·42	0·44, 0·44	0·45	0·44
0·06	0·50	0·44, 0·46	0·47	0·55

It is obvious that estimations carried out with solutions containing 0·10 mg. or more of glycuronic acid will give unsatisfactory results and will lead to the conclusion that the naphthoresorcinol test is unsuitable for quantitative estimation. The poor results obtained by Fürth & Peschek [1936] were clearly due to too high a concentration of glycurone; had the authors diluted their solutions some 20 times they would have obtained the desired results. Since concentrations exceeding 0·05 mg. glycuronic acid per sample give varying and non-reproducible results it follows that when carrying out an estimation of glycurone on a solution of unknown strength a single determination is insufficient and it is first necessary to determine a dilution of the solution to be examined which would bring it within the limits of the method. The test of this is proportionality between concentration and colorimetric value.

When dealing with a solution of unknown concentration samples of 2·0, 1·0 and 0·5 ml. are taken, all made up to 2·0 ml. and treated as described above. If the amount of glycuronic acid lies between 0·005 and 0·05 mg. per sample the readings will represent values in the ratio 2·0 : 1·0 : 0·5. If there is no such proportionality then the solution examined must be diluted 5 or 10 times or more and the procedure repeated.

To illustrate this the following example may be given. A solution containing 80 mg. glycuronic acid in 100 ml. was tested without previous knowledge of the

concentration. Three tests were made: (*a*) with the original solution, (*b*) after diluting 10 times and (*c*) after diluting 100 times, and samples of 2·0, 1·0 and 0·5 ml. were taken from each. After boiling with acid and naphthoresorcinol a precipitate was formed in (*a*) and also in some of the (*b*) samples. For photometric estimation the (*b*) and the (*c*) samples were taken and the following values of E were obtained:

	2·0 ml.	1·0 ml.	0·5 ml.
(*b*) 1 : 10	0·34	0·34	0·35
(*c*) 1 : 100	0·145	0·075	0·035

Only in the (*c*) series is the reading proportional to the amount of fluid taken and therefore only these values can be used for estimation. The coefficient 0·145 corresponds to 0·165 mg. glycuronic acid (see Tables 1 and 2). Taking into account the amount of fluid used for each test and the dilution of 1 : 100, the values obtained are 82·5, 85·0 and 80·0 mg. in 100 ml. (mean value 82·5 mg.), whereas in reality the solution contained 80·0 mg. in 100 ml.

Experiments on these lines have convinced me that it is possible to use this test for the estimation of pure aqueous solutions of glycuronic acid and its conjugated derivatives, but only strictly within the limits of concentration of 0·005–0·05 mg. glycuronic acid per sample, because only in this range of concentrations is there proportionality between absorption and concentration.

Estimation of glycuronic acid in blood and urine

(*a*) *In blood.* Pig's blood was deproteinized with tungstic acid, and with the filtrate corresponding to a tenfold dilution of blood the naphthoresorcinol test gave the following extinction values:

	2·0 ml.	1·0 ml.	0·5 ml.
E	0·17	0·09	0·04

Within the limits of the method these values are proportional and correspond to a content of 9–10 mg. glycuronic acid in 100 ml. blood. In another experiment benzoylglycuronate was added to the filtrate in a quantity representing 60 mg. glycuronic acid per 100 ml. blood. The estimation was carried out in two series, (*a*) diluted 1 : 25 and (*b*) diluted 1 : 50.

	2·0 ml.	1·0 ml.	0·5 ml.
(*a*) 1 : 25	0·50	0·26	0·14
(*b*) 1 : 50	0·24	0·12	0·07

Both series show proportionality between concentration and readings; from the readings a content of 68–80 mg. can be calculated (mean value 74 mg.) per 100 ml. blood. Since there were about 10 mg. present in the blood itself and 60 mg. were added, 70 mg. were to be expected and 74 mg. were actually found.

Equally good results were obtained when benzolyglycuronate was added to the blood directly and the proteins afterwards removed with tungstate. Benzoylglycuronate corresponding to 40 mg. glycuronic acid in 100 ml. blood was added and after removal of proteins the following readings were obtained:

	2·0 ml.	1·0 ml.	0·5 ml.
(*a*) 1 : 25	0·38	0·20	0·10
(*b*) 1 : 50	0·20	0·09	0·05

The above values correspond to 51–57 mg. (mean value 54 mg.) in 100 ml. which is in good agreement with the amount of added glycuronic acid together with that of the blood.

These results prove that the method is suitable for quantitative purposes, provided that the points made with regard to proportionality between the readings and the amount of fluid examined are strictly observed. There are, however, factors in blood which seriously interfere with the naphthoresorcinol test. It was found for instance that the same blood with which satisfactory results were obtained was no longer suitable after haemolysis had occurred owing to storage in the cold for a few days. To such a sample of blood benzoylglycuronate was added in an amount corresponding to 80 mg. glycuronic acid in 100 ml. blood. The following experimental series was prepared: (*a*) blood alone, protein-free filtrate diluted 1 : 10, (*b*) blood with benzoylglycuronate, filtrate 1 : 25 and (*c*) as (*b*) but diluted 1 : 50; the following results were obtained:

	2·0 ml.	1·0 ml.	0·5 ml.
(*a*)	0·10	0·08	0·04
(*b*)	0·43	0·26	0·16
(*c*)	0·27	0·15	0·08

There is a lack of proportionality in all three series, the values nearest to those expected being obtained in the more diluted samples, i.e. those which contain the smallest amount of interfering substances. This example again illustrates the principle that true values can only be obtained when there is proportionality within a given series.

(*b*) *In urine.* The same rule concerning the proportionality between the intensity of colour and the amount of fluid taken applies to estimations of glycuronic acid in urine. In certain urines and especially in concentrated urines, e.g. after excessive sweating, interfering substances occur, and only after 100–200-fold dilution is their effect sufficiently reduced to allow the estimation of added glycuronic acid derivatives. Thus in those cases where the urine contains comparatively large quantities of conjugated glycuronides fairly accurate results will be obtained with suitable dilution. But great obstacles will be met with in studying the excretion of rather small quantities of conjugated glycuronic acids in concentrated urines. Normal urines are usually suitable for the method. Two examples are given below, one with normal urine and another in which interfering substances prevent an accurate estimation of glycuronic acid.

Example I. Four solutions were prepared: (*a*) urine diluted 1 : 10, (*b*) urine diluted 1 : 25, (*c*) urine with added glycurone (40 mg. in 100 ml. urine) diluted 1 : 25, (*d*) as (*c*) but diluted 1 : 50.

The following readings were obtained:

	2·0 ml.	1·0 ml.	0·5 ml.
(*a*)	0·77	0·41	0·21
(*b*)	0·35	0·19	0·09
(*c*)	0·59	0·32	0·17
(*d*)	0·30	0·15	0·08

In all series there is satisfactory proportionality (except with the 2·0 ml. samples in series (*a*) and (*c*), which contain too large amounts of glycuronic acid); the amount of glycuronic acid calculated from series (*a*) and (*b*) is 46–54 mg. (mean value 50 mg.) glycuronic acid in 100 ml. urine. The total amount of preformed and added glycuronic acid, calculated from series (*c*) and (*d*), is 85–97 mg. (mean value 91 mg.) in 100 ml. urine, i.e. 41 mg. more than the amount preformed. As the amount added was actually 40 mg. the result of the estimation may be regarded as satisfactory.

Example II. Six solutions were prepared: (*a*) urine diluted 1 : 25, (*b*) urine diluted 1 : 50, (*c*) urine diluted 1 : 100, (*d*) urine with added glycurone (100 mg. in 100 ml.) diluted 1 : 25, (*e*) as (*d*) but diluted 1 : 50 and (*f*) as (*d*) but diluted 1 : 100.

The following readings were obtained:

	2·0 ml.	1·0 ml.	0·5 ml.
(*a*)	0·14	0·10	0·07
(*b*)	0·11	0·08	0·07
(*c*)	0·08	0·07	0·02
(*d*)	0·34	0·24	0·17
(*e*)	0·26	0·18	0·12
(*f*)	0·18	0·11	0·05

In this example there is no proportionality and no result can be obtained. A very large number of experiments carried out on urine leads to the conclusion that urines occur fairly frequently which give results of this kind. If one were to dissolve in such urines known amounts of glycuronic acid derivatives there would be no proportionality unless such an excess was added that the test was still distinctly positive after dilution to 1 : 100 or 1 : 200. At such degrees of dilution the effect of interfering substances present in some urines is practically abolished. It follows therefore that with urine the estimation as outlined above is only applicable when the effect of disturbing substances is diminished by large dilutions; such dilution is practicable only if the urine contains fairly large quantities of glycuronic acid.

Summary

1. The naphthoresorcinol test of Tollens is suitable under certain conditions for the quantitative determination of glycuronic acid in pure solution. The modification used by Maughan *et al.* [1938] for the estimation of pregnandiol-glycuronic acid was tested on solutions of glycuronic acid, glycurone, benzoyl-glycuronic and bornylglycuronic acids with satisfactory results. It was found however that if the sample examined contains more than 0·05 mg. glycuronic acid variable results are obtained. Single estimations cannot be relied on and series of estimations at varying dilutions are therefore necessary; only the series which shows proportionality between the concentration of glycuronic acid and the intensity of colour is used.

2. The method can be applied to blood and urine but account must be taken of interfering substances. Errors can be avoided by serial estimations, choosing only the series which shows proportionality. In urines which contain interfering substances the estimation can only be carried out if the concentration of glycuronic acid is sufficiently high to allow a considerable dilution.

The author is indebted to the National Culture Fund, Warsaw, for placing the Pulfrich photometer at his disposal.

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100. THE METABOLISM OF PYRUVATE IN PIGEON LIVER

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(*Received 22 April 1940*)

It has been recently demonstrated [Krebs & Eggleston, 1940] that the oxidation of pyruvate by pigeon breast muscle occurs by way of the citric acid cycle. In certain other tissues, such as brain [Banga *et al.* 1939] and liver [Elliott *et al.* 1937], the available data suggest mechanisms for pyruvate removal different from that in muscle, and it seems desirable, therefore, to investigate in detail the differences in pyruvate oxidation in various animal tissues.

The present report deals with the utilization of pyruvate by minced pigeon liver. It was found that in this tissue the reactions comprising the citric acid cycle are present and that pyruvate is probably oxidized by way of the cycle when its concentration is low. In addition, two further reactions involving a rapid removal of pyruvate can occur in this tissue. The first of these, the formation of acetoacetate from pyruvate, has been known for some time [Embden & Oppenheimer, 1913; Annau, 1934; Edson, 1935]. The second reaction which has not been previously demonstrated in liver, is a synthesis of α-ketoglutarate from pyruvate. In contrast to muscle, the synthesis in liver does not require the addition of a C_4 dicarboxylic compound.

Procedure and Methods

In general, the procedure used in the present experiments were similar to those described in previous papers from this laboratory [see Krebs & Eggleston, 1940].

The liver from well-fed pigeons was removed immediately after death and minced in a small hand mincer of the "Fischer" type since the amount of tissue from a single pigeon was insufficient for the use of the "Latapie" mill available. The state of subdivision of the minced tissue is similar to that obtained with the latter apparatus. For each g. of wet tissue, 6·5 ml. of ice-cool, Ca-free phosphate saline [Krebs & Eggleston, 1940] were added. 3 ml. of the suspension were pipetted into conical Warburg vessels and further additions of substrates, water etc. arranged to give a final volume of 4 ml.; thus each ml. of the final suspension contained 100 mg. (wet wt.) of tissue. Preliminary experiments indicated that these conditions gave optimal respiration. Pyruvate is utilized by the suspensions at a rate equal to that of sliced pigeon liver. The use of the minced tissue in the present experiments was preferred since it permits the use of relatively larger quantities of tissue with consequent increased experimental effects.

In the aerobic experiments the vessels were filled with O_2 and NaOH was placed in the centre cups: in the anaerobic ones the gaseous phase was N_2 and the centre cups contained yellow phosphorus. The temperature of the bath in all experiments was 40°. Substrates were usually added from the side arm of the vessels after an equilibration period of 10 min. Malonate, however, was added directly to the liver suspension in order to eliminate the induction period preceding its inhibiting effect. Acidic substances were added as neutral Na salts.

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In those experiments in which α-ketoglutarate and succinate were determined, the vessels were removed from the bath at the end of the experimental period, 2 ml. sodium tungstate added and the solution washed into a measuring cylinder and quickly diluted to 20 ml. with water. 2 ml. 0·66*N* H_2SO_4 were immediately added and the protein precipitate was then filtered off. The determinations were carried out on an aliquot of the filtrate.

A similar procedure was followed when citrate was estimated, except that trichloroacetic acid (5 ml. 7% per 4 ml. tissue suspension) was used to deproteinize the solutions.

When pyruvate was to be measured, the reaction was stopped at the end of the experimental period by the addition of 1 ml. 3*M* acetate buffer of *p*H 5 for each 4 ml. of suspension. The contents of the vessel were filtered and an aliquot of the filtrate used for the determination. Pyruvic acid was determined by the carboxylase method. The respiratory quotient was measured by the method of Warburg & Yabusoe [1924]. Succinic acid was determined manometrically [Szent-Györgyi & Gözsy, 1935; Krebs, 1937] after ethereal extraction from the tungstate-deproteinized solution. Citric acid was determined by the method of Pucher *et al.* [1936]. Acetoacetic acid was estimated by the aniline reaction of Ostern [1933] as modified by Edson [1935]. Fumaric acid was reduced to succinate and determined as the latter; the details of this method will be published shortly.

In earlier experiments α-ketoglutarate was determined directly in the deproteinized solutions by the method of Krebs & Eggleston [1940] in which the acid is oxidized to succinate by treatment with $KMnO_4$ and $MnSO_4$ at 100° for 5 min. Since glutamic acid, if present, yields small but appreciable quantities of succinate under this treatment the procedure was checked by first extracting the acidified deproteinized solution with ether and subjecting the ether-soluble material to permanganate oxidation. The values thus obtained agreed satisfactorily with those from experiments in which the deproteinized solutions were treated directly with the oxidizing mixture. In latter experiments oxidation was performed at room temperature (20°) for a period of 1 hr. Under these conditions, the formation of succinic acid from sources other than α-ketoglutarate is negligible.

I. *The utilization of pyruvate by pigeon liver*

Aerobic utilization of pyruvate. The disappearance of added pyruvate from minced pigeon liver suspended in a Ca-free phosphate-saline medium is extremely rapid. The rate is indicated in the experiment in Table 1, in which 400 mg. wet

Table 1. *Pyruvate and O_2 utilization in minced pigeon liver*

4 ml. liver suspension; O_2; initial pyruvate, 2860 μl. in 4 ml.

Period of incubation (min.)	O_2 used (μl.)	Pyruvate in medium (μl.)	Pyruvate used (μl.)
20	800	1300	1560
40	1590	440	2420
80	2925	207	2653
120	3765	185	2675

tissue (80 mg. dry wt.) are using pyruvate at the rate of 14 mg. per hr. The O_2 uptake of the liver suspensions is not usually increased by the addition of pyruvate but since pyruvate disappears at a much slower rate in the absence of O_2 (see Table 3) part of the O_2 consumed must be concerned with the removal

of pyruvate. It follows that the oxidation of those substrates responsible for the normal respiration of the minced tissue is inhibited by the addition of pyruvate. The ratio of O_2 consumed to pyruvate disappearing is 0·6, indicating incomplete oxidation of the pyruvate. This is likewise suggested by the R.Q. of the reaction being 1·4 (Table 2). Under similar conditions pigeon breast muscle completely oxidizes pyruvate to CO_2 and H_2O, the O_2/pyruvate ratio being 2·5 and the R.Q. 1·2.

Table 2. *Respiratory quotient in the presence of pyruvate*

4 ml. liver suspension; O_2; 30 min.; method of Warburg & Yabusoe [1924]; initial pyruvate concentration, 0·03 *M*.

μl. pyruvate used	1948
μl. O_2 used	1183
μl. CO_2 formed	1673
R.Q.	1·41
Ratio $\frac{O_2 \text{ used}}{\text{Pyruvate used}}$	0·60
Ratio $\frac{CO_2 \text{ formed}}{\text{Pyruvate used}}$	0·85

The effect of malonate. In pigeon muscle, the presence of *M*/400 malonate almost completely inhibits the utilization of pyruvate. Whilst in liver, high concentrations of malonate (*M*/40) cause a slight and possibly non-specific inhibition of pyruvate utilization, *M*/400 and *M*/4000 malonate are without appreciable effect either on the O_2 consumption or pyruvate utilization (Table 3).

Table 3. *Effect of malonate on O_2 and pyruvate utilization*

4 ml. liver suspension; pyruvate (initial concentration 0·03 *M*) added from side arm after 10 min.; malonate added directly to tissue; period of incubation 40 min.

Final concentration of malonate (*M*)	Gas	O_2 used (μl.)	Pyruvate used (μl.)
—	O_2	1309	1995
0·025	O_2	785	1548
0·0025	O_2	1259	2091
0·00025	O_2	1370	2019
—	N_2	—	376
0·025	N_2	—	225
0·0025	N_2	—	406
0·00025	N_2	—	396

If the inhibiting effect of malonate is due to its inhibition of succinic dehydrogenase, it follows that under the conditions of these experiments neither the utilization of pyruvate nor the uptake of O_2 by minced liver requires the activity of succinic dehydrogenase.

The effect of fumarate. The addition of fumarate together with pyruvate to minced pigeon liver has little effect on the O_2 uptake or on the utilization of pyruvate. In the presence of malonate a similar result is obtained (Table 4). These results are, again, in contrast to those obtained with muscle where the addition of fumarate to malonate-poisoned tissue causes the disappearance of an amount of pyruvate equivalent to the added fumarate and an increase in the consumption of O_2 of 2–3 molecules for each molecule of pyruvate disappearing.

Table 4. *Effect of fumarate on pyruvate utilization by pigeon liver*

4 ml. liver suspension; fumarate and pyruvate added from side arm after 10 min.; malonate added immediately to suspension; O_2; period of incubation, 40 min. after addition of pyruvate.

Exp.	Additions	O_2 used (μl.)	Pyruvate used (μl.)
1	0·03 *M* pyruvate	1617	2170
	0·03 *M* pyruvate 0·01 *M* fumarate	1700	1948
2	0·0025 *M* malonate	792	—
	0·0025 *M* malonate 0·03 *M* pyruvate	1122	1643
	0·0025 *M* malonate 0·03 *M* pyruvate 0·01 *M* fumarate	1169	1598
	0·0025 *M* malonate 0·01 *M* fumarate	1150	—

The formation of acetoacetate and α-ketoglutarate. From the data already presented it is clear that the utilization of pyruvate by minced pigeon liver *under the conditions of the present experiments* is unlike the process that occurs in muscle. The question therefore arises as to the details of the process of pyruvate utilization in this tissue. Since during the initial stages of the reaction, the oxidation of pyruvate is incomplete (as indicated by the O_2/pyruvate ratio of 0·6) it is to be expected that intermediate metabolites appear in the suspensions. In fact, two substances which must be considered as products of the incomplete oxidation of pyruvate were found to accumulate in pigeon liver suspensions under the conditions of these experiments: acetoacetate and α-ketoglutarate.

The formation of acetoacetate from pyruvate by liver has been previously described [Embden & Oppenheimer, 1913; Annau, 1934; Edson, 1935]. In the experiments with minced tissue $Q_{\text{acetoacetate}}$ is of the same order of magnitude as found with sliced tissue. In the presence of malonate (Table 7), there is an increase and on the addition of fumarate a decrease in the amount of acetoacetate present. This is in agreement with the earlier results of Annau [1935].

The synthesis of α-ketoglutarate from pyruvate by pigeon liver has not been previously described although the ability of this tissue to synthesize glutamine from ammonium pyruvate suggested that α-ketoglutarate was formed as an intermediate [Örström *et al.* 1939]. When pigeon liver is incubated aerobically with pyruvate, α-ketoglutarate rapidly appears as illustrated in Table 5 where

Table 5. *Synthesis of α-ketoglutaric acid in pigeon liver*

4 ml. suspension of liver; initial pyruvate concentration 0·03 *M*; O_2.

Time (min.)	O_2 used (μl.)	Pyruvate used (μl.)	α-Ketoglutaric acid found (μl.)
20	800	1560	227
40	1590	2420	284
80	2925	2653	287
120	3765	2675	203

$Q_{\text{ketoglutarate}}$ is +8·5 (calculated for the first 20 min. period). After 40 min. the quantity of α-ketoglutarate found remains approximately constant. That this may be interpreted as an "equilibrium" between the synthetic reaction and oxidative removal of the acid follows from the fact that the oxidation of α-ketoglutarate can occur at a rate ($Q_{\text{ketoglutarate}} = -10{\cdot}5$, as calculated from

the data in Table 6) equivalent to its rate of synthesis. When pyruvate and α-ketoglutarate are added together to pigeon liver the utilization of both substrates is inhibited (Table 6), probably as a result of competition between the

Table 6. *α-Ketoglutarate synthesis and disappearance in pigeon liver*

4 ml. suspension liver; O_2; period of incubation 40 min.

Additions	O_2 used (μl.)	Pyruvate used (μl.)	α-Ketoglutarate found (μl.)	α-Ketoglutarate used (μl.)
—	967	—	65	—
0·03 *M* pyruvate	1172	2187	211	—
0·03 *M* α-ketoglutarate	1116	—	782	562
0·03 *M* pyruvate + 0·03 *M* α-ketoglutarate	973	1408	929	415

two substrates for the available oxidation mechanisms. This inhibition of ketoglutarate oxidation by pyruvate may be regarded as responsible for the accumulation of relatively large quantities of α-ketoglutarate under the conditions of these experiments.

Anaerobic formation of α-ketoglutarate. In some experiments a definite anaerobic synthesis of α-ketoglutarate in the presence of pyruvate was observed but the effect was small, varied in magnitude and did not regularly occur.

Formation of carbohydrate from pyruvate. The ability of liver tissue to synthesize carbohydrate from pyruvate is generally recognized. A conversion of pyruvate into carbohydrate occurring simultaneously with the complete oxidation of an additional quantity of pyruvate would account for the O_2/pyruvate ratio found in the present experiments. However, measurements of the total carbohydrate content of the liver suspensions with and without added pyruvate showed no significant differences.

Formation of acetoacetate and α-ketoglutarate in the presence of malonate. The rapid oxidation of α-ketoglutarate makes it impossible to estimate the extent to which ketoglutarate formation accounts for the pyruvate which has disappeared. It is possible, however, to prevent the further oxidation of succinate, the primary product of ketoglutarate oxidation, by the addition of malonate. In these circumstances, measurement of the amount of ketoglutarate plus succinate formed serves as an indication of the quantitative importance of ketoglutarate synthesis in the utilization of pyruvate by pigeon liver. Such an experiment is presented in Table 7. Whilst malonate ($M/400$) has no effect on the quantities of pyruvate or O_2 consumed, increased quantities of both acetoacetate and of ketoglutarate plus succinate are found. On the assumption that each molecule of acetoacetate and of ketoglutarate and succinate require two molecules of pyruvate for their formation, the bulk of the pyruvate disappearing can be accounted for in terms of these two metabolites. In the experiment of Table 7, 63% of the added pyruvate can be accounted for as ketoglutarate and succinate and an additional 28% as acetoacetate. These calculations, it should be noted, are made on the assumption that an appreciable formation of ketoglutarate, succinate and acetoacetate does not occur from sources other than pyruvate when this substance is present in large excess.

Since the rate of pyruvate utilization by liver is not affected by malonate it is probable that the two reactions (α-ketoglutarate and acetoacetate synthesis) which can be demonstrated as accounting for the bulk of the pyruvate used by malonate-poisoned tissue, are of similar quantitative importance in the oxidation of pyruvate by liver tissue in the absence of malonate. It follows then that

Table 7. *Formation of acetoacetic acid and α-ketoglutaric acid in the presence of malonate*

4 ml. liver suspension; pyruvate added from side arm after 10 min., malonate added directly to tissue; O_2; period of incubation 30 min.

Additions	O_2 used (μl.)	Pyruvate used (μl.)	Acetoacetic acid formed (μl.)	Ketoglutaric + succinic acid found (μl.)
0·025 *M* malonate	580	—	34	164
0·03 *M* pyruvate	1687	1605	38	233
0·025 *M* malonate + 0·03 *M* pyruvate	952	1640	233	518

the observed differences between pyruvate utilization in muscle and in liver are due largely to the presence of these additional reactions in liver. Whilst muscle is incapable of synthesizing appreciable quantities of the dicarboxylic compounds used in its metabolism, the liver is capable of effecting such a synthesis.

II. *The citric acid cycle in pigeon liver*

Oxidation and synthesis of citrate. In the preceding section it has been shown that α-ketoglutarate can arise directly from pyruvate in pigeon liver. The further oxidation of α-ketoglutarate would lead to oxaloacetate and this substance may possibly be further metabolized, as in muscle, by condensing with additional pyruvate to form citrate, i.e. by the citric acid cycle. The presence of the citric acid cycle in pigeon liver would require that the tissue should be capable of oxidizing citrate at a rate at least one-sixth that at which O_2 was being consumed and also that citrate could be synthesized from pyruvate and oxaloacetate. That pigeon liver satisfies both these criteria is demonstrated in Tables 8 and 9. In Table 8 $Q_{citrate}$ is $-14{\cdot}3$ as compared to Q_{O_2} of $-16{\cdot}2$: the oxidation of citrate

Table 8. *Oxidation of citrate in pigeon liver*

4 ml. liver suspension; O_2.

Exp.	Additions	Time (min.)	O_2 used (μl.)	Citrate found (μl.)	Citrate used (μl.)
1	Citrate (1344 μl.)	30	648	700	644
2	Citrate (1344 μl.)	60	1290	200	1144

Table 9. *Anaerobic formation of citrate in pigeon liver*

4 ml. liver suspension; N_2; period of incubation 60 min.

Additions	Pyruvate found (μl.)	Pyruvate change (μl.)	Succinate found (μl.)	Ketoglutarate found (μl.)	Citrate found (μl.)
2604 μl. pyruvate	2095	− 509	106	75	4
2604 μl. pyruvate + 4480 μl. oxaloacetate	3840	+1236	121	349	373
2604 μl. pyruvate + 2240 μl. oxaloacetate	2560	− 44	97	343	126

occurs at a rate far more rapid than that required by theory. Table 9 illustrates the anaerobic synthesis of citrate in pigeon liver from oxaloacetate and pyruvate. $Q_{citrate}$, which in the presence of pyruvate alone is very small, is increased to about 7 in the presence of $M/20$ oxaloacetate and to about half this amount when the oxaloacetate concentration is $M/40$. Together with citrate, α-ketoglutarate

is formed under these conditions in relatively large amounts. From analogy with muscle, one may conclude that this arises from the oxidation of citrate to α-ketoglutarate by oxaloacetate. The rate of citrate synthesis, as calculated in these experiments from the quantity of citrate found, is therefore minimal and a more precise estimation of the rate of citrate synthesis should be based on the amounts of citrate plus α-ketoglutarate formed.

In muscle, the synthesis of citrate from oxaloacetate and pyruvate as well as the oxidation of citrate to ketoglutarate is coupled with the reductive formation of one mole of malate [Krebs, 1940, 1, 2]. If a similar reaction occurred in liver, therefore, one would expect to find a quantity of malate and fumarate formed, equal to one mole for every mole of citrate produced plus two moles of malate for each mole of ketoglutarate. In Table 10 an experiment in which oxaloacetate alone was added indicates that malate and fumarate are formed in quantities of the magnitude expected. In this experiment the quantity of oxaloacetate added was small and little citrate was found, the bulk of the citrate having been further oxidized to ketoglutarate. The quantity of malate plus fumarate necessary to account for the citrate and ketoglutarate formed should be, as calculated from the above considerations, 405 μl., whilst 580 μl. were found.

Table 10. *The fate of oxaloacetic acid in pigeon liver*

4 ml. liver suspension; N_2; period of incubation 40 min.

	μl.		
Oxaloacetate added	1344	= 0·240	milliequiv. C added
CO_2 and HCO_3' formed*	1285		
Ketoglutarate found	197		
Pyruvate found	103		
Fumarate found	121	580	= 0·234 milliequiv. C recovered
Malate (calculated)†	459		
Succinate found	61		
Citrate found	11		

* Method of Warburg & Yabusoe [1923]. Corrected for CO_2 evolved from tissue alone and for HCO_3' from oxaloacetate.

† *l*-Malate/fumarate ratio = 3·8 [see Krebs, 1940, 2]

About 97 % of the carbon added in the form of oxaloacetate can be accounted for by the products listed in Table 10, which thus represents a fairly complete balance sheet.

It is clear then, that liver tissue is capable of removing pyruvate by the citric acid cycle when oxaloacetate is present. It follows that α-ketoglutarate can arise in liver tissue in two ways, as a result of the oxidation of citrate or by formation from pyruvate without added C_4 dicarboxylic compounds. There are, therefore, two cyclic processes in liver, the citric acid cycle, and another cyclic series of reactions similar to that discussed by Toeniessen & Brinkmann [1930] and by Elliott *et al.* [1937]

(1) 2 pyruvate → ketoglutarate → succinate → fumarate → oxaloacetate → pyruvate + CO_2.

According to the latter scheme oxaloacetate reacts by way of decarboxylation whilst in the citric acid cycle it condenses with pyruvate to yield citrate. Data in reference to the relative importance of the two cycles have already been presented in Table 9. If oxaloacetate according to scheme (1) was entirely decarboxylated to pyruvate then the effect of oxaloacetate should be simulated by the addition of an appropriate quantity of pyruvate. As illustrated in Table 9, however, the anaerobic formation of ketoglutarate and citrate from oxaloacetate is much larger than that found in the presence of pyruvate alone, in this

101. STUDIES IN SYNTHETIC IMMUNO-CHEMISTRY

5. OBSERVATIONS ON ANTISERA AGAINST ASPIRIN-PROTEIN COMPLEXES

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With a note on the serological reactions by A. A. Miles

(*Received 16 April 1940*)

In a recent paper [Clutton *et al.* 1938, 2] it was shown that antisera against protein complexes in which thyroxine was the immunologically determinant group were capable of producing a state of resistance against the normal physiological effects of thyroxine in another animal. It was suggested at the time that this observation might represent a general phenomenon, although it was pointed out that such a generalization could not safely be made from the experiments with thyroxine alone; the latter compound is an amino-acid, and it is uncertain whether it is active in the body in the form of a protein; if such were the case the neutralization of the action of thyroxine by the antisera against thyroxyl proteins might be no different from an ordinary toxin-antitoxin neutralization.

In order to obtain clearer evidence it was therefore desirable to see whether similar pharmacological neutralization could be effected with antisera against proteins containing immunologically determinant groups which were pharmacologically active but not chemically allied to proteins.

For the purpose of this investigation the group selected was the acetylsalicyl residue; the reasons for this choice were (*a*) the probability that this residue would act as a powerfully determinant group in the immunological sense and (*b*) the fact that with the aid of the method recently developed by Brownlee & Gaddum [1939] aspirin can be assayed with reasonable accuracy.

The protein complexes required for the experiments were obtained as follows. Aspirin was first converted into acetylsalicyl chloride; this, on treatment with sodium azide, gave acetylsalicyl azide, which, when brought into reaction with serum globulin in alkaline solution, yielded a product containing about 5% of aspiryl residues. Such products proved, as will be seen below, to be powerful antigens, and moreover inhibition reactions indicated that their immunological properties were specifically determined by the acetylsalicyl residue. It will be noted that in these complexes, as in those described in previous papers, the haptene group introduced is combined in amide linkage with the free amino-groups of the protein; we have already shown [Clutton *et al.* 1938, 1; Humphrey & Yuill, 1939] in the case of the β-glucosidotyrosyl residue that groups attached in this particular manner have a very powerful effect in masking the original immunological specificity of the protein.

Having thus obtained strongly reacting antisera against aspirin-protein complexes of the type described above, we proceeded to investigate their

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physiological properties in the following manner. A number of young male rats of similar weight and age were divided into two groups; the first (control) group received a course of injections of normal rabbit serum and the second a similar course of injections of the antiserum; pyrexia was then produced in both groups by the injection of equal amounts of a suspension of yeast; the effect of a given dose of aspirin in lowering the high temperature thus produced was then measured according to the technique of Brownlee & Gaddum [1939].

It will be seen from the results which are recorded below that in 6 experiments performed in this manner the antipyretic effect of aspirin has been constantly less in the animals treated with the antiserum against the aspiryl-protein than in the animals similarly treated with normal rabbit serum. In 4 out of the 6 experiments the differences observed between the two groups have high degrees of statistical significance; in the remaining two the differences are in the same direction but are too small to justify the drawing of any conclusions; taking the results from each experiment separately and from all the experiments together, however, it is reasonable to conclude that the effect of the antiserum is definitely established.

We feel satisfied therefore that the present work demonstrates the possibility of neutralizing the effect of a pharmacologically active group with an antiserum against a protein in which this group is acting as a haptene, even though the group in question has no chemical connexion with protein itself. If this conclusion be accepted the results recorded in the present paper, together with those of our previous work [Clutton *et al.* 1938, 1] must be regarded as examples of a general phenomenon which may be described as an *in vitro* counterpart of the serological inhibition reaction. In the latter reaction occupation of the specific combining sites on the antibody molecule by the haptene prevents the combination of antigen and antibody from taking place: in our experiments, on the other hand, it appears that attachment of the haptene to the antibody is preventing the former from reaching its normal site of action in the body and therefore from exercising its usual physiological effects.

An extension of experiments of this type to protein complexes containing physiologically active steroids as haptene groups is now in hand.

Experimental

Preparation of compounds

Acetylsalicyl chloride. Several methods have been described in the literature for the preparation of this compound; in our hands however none of these proved very satisfactory and we obtained better results as follows. Aspirin (20 g.), phosphorus pentachloride (25 g.) and anhydrous chloroform (40 ml.) were placed in a Claisen flask and shaken with ice-cooling until all the aspirin had dissolved. The reaction mixture was kept at room temperature for 15 min. and then evaporated under diminished pressure with a bath temperature of 100°; on raising the bath temperature to 160–170° the acetylsalicyl chloride distilled (B.P. 140°/15 mm.) as a yellow viscous oil which was preserved in sealed tubes.

Acetylsalicyl azide. A solution of acetylsalicyl chloride (1 g.) in anhydrous ether (10 ml.) was placed in a separating funnel together with 2·5 ml. water containing 0·5 g. sodium azide. The funnel was shaken for 15 min., after which the aqueous layer was discarded. The ethereal layer was washed twice with water, dried over anhydrous Na_2SO_4 and evaporated under a current of N_2 without application of heat; the oily residue solidified on rubbing to a mass of white crystals having M.P. 56–58° with decomposition.

For characterization this compound was converted into the corresponding *benzyl urethane*: the freshly prepared azide (from 1 g. acetylsalicyl chloride) was dissolved in benzyl alcohol (1·6 ml.) and the solution was heated on a steam bath until evolution of N_2 had ceased (20 min.); the solution was chilled, mixed with light petroleum (10 ml. of B.P. 100–120°) and left in the ice-chest overnight; next day the supernatant liquid was decanted from the crystalline precipitate which had formed and the solid was collected with the aid of more petroleum. The yield was 1·1 g. After recrystallization from absolute alcohol the substance formed colourless silky needles, M.P. 74–76°. (Found: N, 4·8, 4·9%; $C_{16}H_{15}O_4N$ requires N, 4·9%.)

Coupling of acetylsalicyl azide with serum globulin. Preparations were made from both horse and rabbit serum globulins; a typical experiment was as follows. Horse serum globulin solution (52 ml.) containing 1·25% N was diluted with an equal volume of water and made alkaline to phenolphthalein; dioxane (104 ml.) was added with stirring and the resulting clear solution was chilled in ice. Acetylsalicyl azide (0·82 g.) dissolved in dioxane (5 ml.) was added to the protein solution with continued stirring over about 20 min.; stirring was maintained for another 20 min. The reaction mixture was kept alkaline to phenolphthalein throughout the experiment by the periodic addition of *N* NaOH of which 3·7 ml. were required in all.

At the end of the reaction the solution was thick and opalescent; it was adjusted to *p*H 4·5 by the addition of 2*N* HCl when a heavy precipitate formed. After keeping for 1 hr. at 0° the mixture was centrifuged and the supernatant liquid discarded. The precipitate was suspended in 250 ml. water and dialysed overnight against running tap water. The sludge from the dialysis bag was diluted with 100 ml. water and the mixture made alkaline to phenolphthalein with *N* NaOH when all the solid dissolved. The solution was rapidly readjusted to *p*H 6·9 with *N* HCl, cleared on the centrifuge and stored in the ice chest with a few crystals of thymol as preservative.

Acetyl determinations (Pregl) carried out on samples of this protein solution which had been evaporated to dryness indicated that the coupled product contained about 5% of acetylsalicyl residues; this figure however must be regarded as only approximate, since the blank correction for the apparent acetyl content of the original protein was proportionately large.

Acetylsalicylglycine. Attempts to demonstrate specific inhibition of the precipitin reactions between the aspirin-protein complexes and their homologous antisera by aspirin itself proved unsatisfactory, since excessively large amounts of inhibitor had to be used to produce any effect. It was suspected that this might be due to the fact that the immunologically determinant character of the haptene group in these products involved not only the acetylsalicyl residue itself but also the combination of this residue in amide linkage; it was therefore decided to repeat these experiments with a simple compound of the latter type, namely acetylsalicylglycine.

This compound was prepared in the following manner. Salicyl hydrazide (3·7 g.) was dissolved in 5*N* HCl (12 ml.); the solution was cooled to 0° and treated with 30% $NaNO_2$ (10 ml.) which was added slowly with mechanical stirring. The mixture was kept at 0° for 10 min. longer, with continued stirring, and the azide which had separated was then collected and washed thoroughly with water on the filter. The precipitate was taken up in ether, the ethereal solution being washed twice with 5% aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4 and filtered. To the solution of the azide thus prepared was added a dry ethereal solution of glycine ethyl ester obtained from 7 g. of the hydro-

chloride. The mixture was kept overnight at room temperature, washed twice with 2*N* HCl, twice with 5% $NaHCO_3$, twice with water and dried over anhydrous Na_2SO_4; the ether was evaporated and the residue crystallized from acetone-light petroleum. *Salicylglycine ethyl ester* separated in long silky needles, M.P. 98–99°; the yield was 4·35 g. (Found: N, 6·21 %; $C_{11}H_{13}O_4N$ requires N, 6·28 %.)

The above ester (1 g.) was stirred with *N* NaOH (9 ml. : 2 equiv.) until all was dissolved and kept for 15 min. at room temperature; the solution was then cooled to 0° and treated with acetic anhydride (0·45 ml.) which was added in three equal portions with vigorous shaking and continued cooling over 15 min. Addition of 2 ml. 5*N* H_2SO_4 and rubbing with a glass rod caused separation of fine colourless needles of *acetylsalicylglycine*; the compound had M.P. 147–149° and the yield was 0·85 g. (Found: N, 5·30 %; equiv. by titration 260; $C_{13}H_{15}O_5N$ requires N, 5·28 %; equiv. by titration 265.) The substance gave no colour with $FeCl_3$.

Immunization experiments

Two batches of antiserum against aspiryl horse serum globulin were prepared as follows. *Batch A*. Each of 15 rabbits received 3 injections of 10 mg., 3 of 20 mg. and 3 of 30 mg. of the protein, the doses being given thrice weekly over 3 successive weeks; after a rest of 1 week 5 further injections, rising in amount from 30 to 50 mg., were given at 2-day intervals. *Batch B*. Each of 11 rabbits received a first course of injections similar to that used in the preparation of Batch A; after a rest of 2 weeks a second course was given, consisting of 3 doses of 30 mg., 3 of 40 mg. and 3 of 50 mg., the injections again being made thrice weekly. In the preparation of both batches the first courses were given by the intravenous route and the second courses intraperitoneally; both groups were bled out 10 days after the final injection.

Ring precipitation tests were carried out, using undiluted antiserum, with aspiryl rabbit serum globulin as test antigen; both batches of antiserum showed precipitation at all dilutions of antigen up to 1 : 100,000, while controls with normal rabbit serum as precipitin were uniformly negative. The precipitations were inhibited by preliminary incubation of the antiserum with an equal volume of a 1 % solution of aspirylglycine. A more precise description of the serological reactions is given in the note by Prof. A. A. Miles at the end of this paper.

Physiological experiments

Young male Wistar strain rats were used for the experiments; except in the first experiment they were kept in a thermostatically controlled room, the temperature of which did not vary more than 4°. The animals were kept on a constant diet for some time before the beginning of the experiment.

Each rat in the experimental group received an injection of 3 ml. of antiserum intraperitoneally on the mornings of 3 successive days while each animal in the control group was simultaneously treated with a dose of 3 ml. of normal rabbit serum. At noon on the third day food was withdrawn from all animals and their weights and temperatures (T_1) were observed; at 5 p.m. on that day each rat received a subcutaneous injection of a 15 % suspension of dried yeast in physiological saline containing 2 % gum acacia; the suspension was freshly prepared and the amount given was 1 ml. per 100 g. body weight.

At 9 a.m. on the next day, when the pyrexia produced by the yeast was at its height, the temperatures of the rats were again recorded (T_2); all animals failing to show a significant rise of temperature at this stage were discarded. Immediately after the observation of T_2 each rat was given the predetermined

dose of aspirin which was administered by stomach tube as the sodium salt dissolved in tap water containing 2% gum acacia; the body temperatures of the animals were recorded at 1 hr. (T_3), 2 hr. (T_4) and 3 hr. (T_5) after the administration of the aspirin.

The sum of the differences (T_2-T_3), (T_2-T_4) and (T_2-T_5) was taken as representing the total fall in temperature produced by the dose of aspirin administered; this figure was determined for each individual rat and its mean value was calculated for the experimental and control groups respectively.

The results of 6 such experiments are summarized in Table 1 and a detailed protocol of a typical one of these experiments is appended to this paper. The observations have been analysed statistically according to Burn [1937, pp. 28–30]; it will be seen from the last column of Table 1 that in 4 out of the 6 experiments the value of P is 0·05 or less, indicating a high degree of significance for the differences observed in these cases. In the remaining 2 experiments the differences, although they lie in the anticipated direction, are not significant.

Table 1. *Summary of results obtained with specific inhibition of the anti-pyrexial action of aspirin*

Exp.		No. of rats	Aspirin mg. per kg. body weight	Anti-serum	Total fall of temperature ° F.	P
1	Controls	7	66	Batch A	4·67	
	Experimentals	6	66	,,	2·40	0·05
2	Controls	9	66	,,	3·45	
	Experimentals	9	66	,,	2·76	0·45
3	Controls	4	132	,,	12·76	
	Experimentals	4	132	,,	7·56	0·05
4	Controls	16	132	,,	5·18	
	Experimentals	19	132	,,	4·27	0·30
5	Controls	9	66	Batch B	3·81	About
	Experimentals	12	66	,,	0·26	0·001
6	Controls	12	66	,,	5·23	
	Experimentals	13	66	,,	2·97	0·01

An attempt to obtain additional evidence by using the "cross-over" type of experiment suggested by Brownlee & Gaddum [1939] failed since, after one injection of yeast, rats become hypersensitive to its pyrexial action on subsequent injections.

Note on the serological reactions of anti-aspiryl horse serum globulin

By A. A. Miles

Immunization. Antisera were prepared in 3 rabbits by a technique similar to that described above for the preparation of antiserum Batch B, each animal receiving a total of approximately 0·36 g. of aspiryl horse serum globulin.

Precipitation reactions. The precipitation reactions of the antisera were studied by the optimal proportions technique of Dean & Webb [1926]; in order to avoid complications due to residual precipitins against normal horse serum proteins the test antigen employed for the detailed investigation was prepared by coupling aspirin with rabbit serum globulin.

At the end of the first course of injections all three antisera precipitated the homologous (horse serum) antigen, but only one precipitated the aspiryl rabbit serum and that in one tube alone. In the homologous antigen-antibody system

precipitation occurred slowly at 20°, rapidly at 37° and not at all at 56°, though precipitates already formed at the lower temperatures were not appreciably dissolved at 56°.

After the second course of injections all three antisera gave wide zones of precipitation, appearing in 1 hr. at 37°, with aspiryl rabbit serum globulin. Titration of falling concentrations of this antigen against equal volumes of 1 : 5 dilution of serum showed that the optimal concentration of antigen was 0·023 %.

Inhibition tests. These were carried out on the optimal system just described as follows: 0·2 ml. volumes of falling concentrations of the various inhibitors were incubated for 1 hr. at 37° with 0·2 ml. quantities of a 1 : 2·5 dilution of antiserum; the resulting mixtures were then tested for precipitation with 0·4 ml. volumes of 0·023 % aspiryl rabbit serum globulin.

The inhibitors studied were neutralized solutions of the sodium salts of aspirin, salicylic acid, aspirylglycine and salicylglycine. Both retardation and total inhibition of precipitation were observed; in some cases low concentrations of inhibitors delayed precipitation for several hours as compared with controls, but in all experiments the systems reached final equilibrium after 6–12 hr. at 37°. The results of the tests are given in Table 2; the readings recorded were taken at 6 hr. and represent the final inhibiting titres, the initial retarding effects being neglected.

Table 2. *Inhibition by various reagents of anti-aspiryl horse serum globulin optimally precipitating 0·23 mg. of aspiryl rabbit globulin*

Inhibitor (neutral solutions)	Amount of inhibitor, mg./0·23 mg. antigen								mg. inhibitor equivalent to 0·23 mg. of aspiryl rabbit globulin
	40·0	20·0	10·0	5·00	2·50	1·25	0·62	0·00	
Aspiryl-glycine	nt	nt	–	–	op	tr	+ +	+ +	2·5
Salicyl-glycine	nt	nt	–	op	op	tr	+ +	+ +	2·5
Na acetyl-salicylate	op	tr	+	+ +	+ +	+ +	+ +	+ +	40·0
Na salicylate	nt	op	tr	+ +	+ +	+ +	+ +	+ +	20·0

op = opalescence; tr, +, + + = trace, and increasing degrees of precipitation; nt = no test.

The inhibiting titres in Table 2 are comparative only. In the precipitating system under discussion the optimal ratio of Dean & Webb [1926] is constant over a wide range of antibody dilutions, i.e. the optimally reacting amounts of antigen and antibody are in constant proportion. There is no similar linear relationship between concentration of reactants in the optimally precipitating system and concentration of inhibitor; thus in one experiment the following results were obtained:

Dilution of serum mixed with optimal amount of antigen	Minimum inhibiting conc. of aspirylglycine in mg./ml.
1 : 2·3	5·0
1 : 4·6	1·9
1 : 9·2	0·3

These figures show that with rising dilution of the precipitating system the effective concentration of inhibitor falls with disproportionate rapidity. Such

deviation from linear relationship may of course be more apparent than real, since in this as in other similar systems the power of the antiserum to precipitate the antigen falls off rapidly with dilution. It must be remembered that the occurrence of antigen-antibody reaction is not necessarily manifested by visible precipitation; that is to say reaction may still be taking place at dilutions beyond the limit at which there is formation of an actual precipitate; it is possible that, as the concentration of the reacting system approaches the limit for precipitate formation, the occupation of combining sites on the antibody molecule by the inhibitor will become relatively more effective in preventing the occurrence of the visible reaction, so that the amount of inhibitor required will appear to be relatively less.

In any event the results in Table 2 show that fairly high and almost equal degrees of inhibition are obtained in this system with aspiryl- and salicyl-glycine whilst aspirin and salicylic acid are relatively ineffective. This shows (*a*) that the immunological properties of the antigen are largely determined by the new group introduced and (*b*) that the determinant is not the benzenoid portion of this group alone but the entire aspiryl- (or salicyl-) amido complex. The fact that inhibition is obtained with salicyl- as well as with aspiryl-glycine may indicate either that the acetyl group of aspirin is relatively unimportant or that the immunological properties of the antigen are partly determined by salicylamido groups as well as by aspirylamido groups; such a situation might well result from the partial deacetylation of the aspiryl protein complex during its preparation.

Summary

Aspiryl protein complexes have been prepared by coupling acetylsalicyl azide with horse- and rabbit-serum globulins.

Immunization of rabbits against the aspiryl horse serum globulin gives rise to strongly reacting antisera, the serological properties of which are specifically determined by the aspiryl- (or salicyl-) amido grouping.

Such immune sera are able partly to protect rats, suffering from pyrexia as the result of injection of yeast, from the normal antipyretic effect of aspirin.

We desire to thank Dr G. Brownlee for advice in connexion with the physiological experiments recorded in this paper. One of us (M. E. Y.) is indebted to the Medical Research Council for a personal grant.

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Protocol of Exp. 6

Rat weight (g.)	T_1 (° F.)	T_2 (° F.)	T_3 (° F.)	T_4 (° F.)	T_5 (° F.)	T_2-T_3 (° F.)	T_2-T_4 (° F.)	T_2-T_5 (° F.)	Total fall (° F.)
			A. Control group: normal serum.						
200	99·4	100·6	99·6	99·2	100·1	1·0	1·4	0·5	2·9
170	99·8	100·7	100·1	98·6	99·2	0·6	2·1	1·5	4·2
190	100·4	101·4	99·6	98·8	99·0	1·8	2·6	2·4	6·8
210	100·8	101·7	99·3	99·8	99·9	2·4	1·9	1·8	6·1
170	99·6	101·3	99·3	99·1	98·8	2·0	2·2	2·5	6·7
170	100·7	101·8	100·8	100·5	100·2	1·0	1·3	1·6	3·9
160	100·8	101·8	99·7	99·4	99·7	2·1	2·4	2·1	6·6
170	99·8	100·8	99·7	99·5	100·4	1·1	1·3	0·4	2·8
200	99·6	101·4	99·7	99·3	99·1	1·7	2·1	2·3	6·1
180	100·0	101·5	100·7	100·5	100·1	0·8	1·0	1·4	3·2
150	100·5	101·8	99·6	99·4	99·5	2·2	2·4	2·3	6·9
200	99·6	101·6	99·4	99·7	99·1	2·2	1·9	2·5	6·6
								Mean (M_1) =	5·23
								ϵ_1^2 =	0·235
			B. Experimental group: anti-aspiryl horse serum globulin						
160	98·6	100·0	100·1	99·1	99·1	−0·1	0·9	0·9	1·7
190	100·5	101·1	101·2	100·5	99·8	−0·1	0·8	1·4	1·9
150	99·4	100·4	100·4	100·5	99·9	0	−0·1	0·5	0·4
190	99·4	101·4	100·5	99·8	100·3	0·9	1·6	1·1	3·6
190	98·8	100·5	100·5	100·0	99·4	0	0·8	1·1	1·6
190	99·9	101·0	100·5	100·4	99·7	0·5	0·6	1·3	2·4
180	99·1	102·0	100·6	100·0	100·1	1·4	2·0	1·9	5·3
180	99·8	101·7	100·7	99·2	99·5	1·0	2·5	2·2	5·7
200	99·0	100·8	100·0	100·2	100·3	0·8	0·6	0·5	1·9
150	99·5	101·6	99·7	99·5	99·7	1·9	2·1	1·9	5·9
210	99·6	101·2	100·5	99·9	100·2	0·7	1·3	1·0	3·0
220	99·6	102·0	100·6	100·1	100·7	1·4	1·9	1·3	4·6
230	99·3	100·0	100·1	100·0	99·3	−0·1	0	0·7	0·6
								Mean (M_2) =	2·97
								ϵ_2^2 =	0·269

N.B. Eight controls and seven experimentals were discarded because of insufficient pyrexia with yeast.

A. Control group:

Total drop for 12 rats over 3 hr. = 62·8° F.

Average drop per rat per reading = 1·74° F.

B. Experimental group:

Total drop for 13 rats over 3 hr. = 38·6° F.

Average drop per rat per reading = 0·99° F.

$$\frac{M_1 - M_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}} = 3{\cdot}19.$$

3·19 corresponds to $P < 0{\cdot}01$. See Burn [1937].

102. THE PRODUCTION OF AMINES BY BACTERIA

2. THE PRODUCTION OF TYRAMINE BY *STREPTOCOCCUS FAECALIS*

By ERNEST FREDERICK GALE[1]

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(*Received 25 April 1940*)

In the first paper of this series, the author [Gale, 1940] showed that washed suspensions of *Bact. coli* will decarboxylate *l*(+)-arginine, *l*(+)-lysine, *l*(+)-ornithine, *l*(−)-histidine and *l*(+)-glutamic acid to form agmatine, cadaverine, putrescine, histamine and γ-aminobutyric acid respectively. Of 14 strains of coliform organisms investigated, 12 decarboxylated arginine, 12 histidine, 13 lysine, 12 ornithine and 9 glutamic acid. The decarboxylases involved proved to be very thermolabile and were formed more easily in organisms grown at 27 than in those grown at 37°. The decarboxylase activity of the organisms was shown to depend upon the *p*H of the medium during growth: organisms grown at *p*H 7 have little decarboxylase activity but this is greatly increased (20–100-fold) by growing the organisms at *p*H 5. In most cases the activities were also increased by the presence of glucose in the growth medium but this increase in activity is due to the fall in *p*H produced during growth by the fermentation acids. The decarboxylases are active over a very restricted *p*H range, the optimum value in each case being: for arginine, 4·0; lysine, 4·5; histidine, 4·0; ornithine, 5·0 and glutamic acid, 4·0. None of the strains investigated decarboxylated any of the common amino-acids, other than the five mentioned, under the experimental conditions used.

Emerson [1902] showed the presence of tyramine in autolysing pancreas, while Rosenheim [1909] and Barger & Walpole [1909] showed it to be one of the main pressor substances in placental extracts and putrefying meat respectively. Sasaki [1914] obtained tyramine from a medium of salts, tyrosine and glycerol heavily inoculated with *Bact. coli* and incubated, while Hanke & Koessler [1924] obtained this amine by the growth of pure cultures of organisms isolated from the intestine in synthetic media containing tyrosine, salts, glycerol and lactose. As a result of their investigations they suggest that organisms producing histamine are incapable of forming tyramine and *vice versa*.

The present communication deals with the production of tyramine from tyrosine by washed suspensions of *Strep. faecalis* and describes the properties of the tyrosine decarboxylase present in certain strains of that organism.

Methods and organisms used

The methods used in this investigation for the preparation of washed bacterial suspensions and the investigation of their decarboxylase activities were essentially the same as those described previously [Gale, 1940]. Of the strains of *Strep.*

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faecalis used, no. 1 is the stock strain of the laboratory; nos. 2–5 were freshly isolated from faeces and given to us by Dr Carruthers of the Cambridge University Pathology Dept. and nos. 6, 7 were obtained from the National Institute of Type Cultures.

Preliminary work

Warburg manometers were set up as follows: in the main compartment, 1 ml. washed suspension of stock *Strep. faecalis* prepared from a 17 hr. culture in 2% glucose broth (tryptic digest of casein) grown at 27°, 1·5 ml. 0·05 *M* phthalate buffer and in the side cup 0·5 ml. *M*/30 amino-acid solution. The manometers were filled with N_2, equilibrated in a thermostat at 30° and the contents of the side cup tipped into the main compartment after equilibration. The following amino-acids were tested at the *p*H values shown—the values chosen being the optimum *p*H in those cases where the decarboxylase has already been studied in *Bact. coli* and *p*H 5·0 in other cases as a probable experimental *p*H.

	*p*H		*p*H
Glycine	5·0	*l*(−)-Aspartic acid	5·0
dl-Alanine	5·0	*l*(−)-Proline	5·0
dl-Valine	5·0	*dl*-Serine	5·0
l(−)-Leucine	5·0	*l*(−)-Cysteine	5·0
l(−)-Tyrosine	5·0	*l*(+)-Arginine	4·0
l(−)-Tryptophan	5·0	*l*(+)-Ornithine	5·0
l(+)-Glutamic acid	4·0	*l*(−)-Histidine	4·0
l(+)-Phenylalanine	5·0	*l*(+)-Lysine	4·5

The only substrate for which any gas evolution occurred ($Q_{CO_2} < 1$ regarded as not significant) was *l*(−)-tyrosine. Repetition of the experiment with 0·3 ml. 20% NaOH in the absorption cup gave no net gas output, showing that the liberated gas is CO_2. At the completion of the experiment, the contents of the cups were tested for NH_3 formation by distillation in Conway vessels followed by nesslerisation, with completely negative results. Controls with organism alone gave negligible CO_2 output or NH_3 formation at *p*H 5·0 under the experimental conditions.

Tyrosine decarboxylase of Strep. faecalis

p*H optimum*. A series of manometers was set up with the usual contents but with phthalate buffers of *p*H 3·5–6·5. The amount of organism used in each cup was 2–3 mg. dry wt. determined with a photoelectric turbidimeter previously calibrated against the organism in question. Fig. 1 shows the variation of the rate of decarboxylation of tyrosine expressed as Q_{CO_2} (= μl. CO_2 liberated/hr./mg. dry wt. of organism) with the reaction *p*H. The optimum value is *p*H 5·0 when $Q_{CO_2} = 244$ for this organism at 30°. The tyrosine decarboxylase thus falls into line with the other amino-acid decarboxylases investigated in being optimally active between *p*H 4·0 and 5·0.

Temperature conditions. The decarboxylation was next investigated at various temperatures; manometers containing the usual quantities at the optimum *p*H of 5·0 being set up in thermostats at 18, 25, 31, 37 and 44° respectively. After the addition of the substrate readings were taken every 10 min. for 2 hr. From the figures obtained the velocity of reaction (expressed as Q_{CO_2}) at various times after the addition of the substrate was calculated; Fig. 2 shows the variation of this velocity with time at various temperatures. The temperature coefficient calculated for maximum velocities attained over the range 20–30° is 2·1. Fig. 2 shows that the velocity fluctuates rapidly for the first 10–20 min. of the reaction before a fairly steady rate is achieved; for con-

venience an experimental temperature of 30° has been adopted as in previous work and Q_{CO_2} values quoted below are calculated from the steady rate attained 15–20 min. after addition of the substrate.

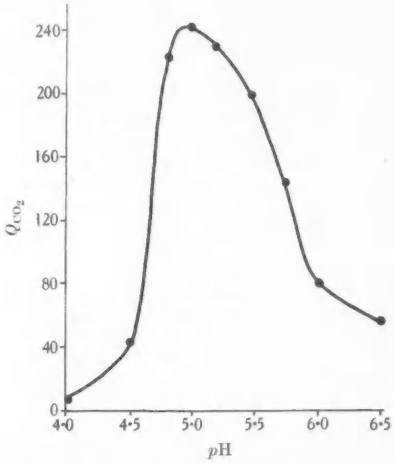

Fig. 1. Variation of tyrosine decarboxylase activity (Q_{CO_2}) with *p*H, *Strep. faecalis*.

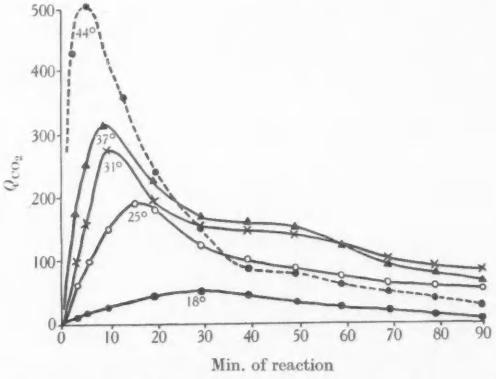

Fig. 2. Tyrosine decarboxylase activity of washed suspensions of stock *Strep. faecalis*: variation of velocity of reaction (expressed as Q_{CO_2}) with time of reaction at different temperatures.

Growth conditions

Growth temperature. With the decarboxylases of *Bact. coli*, it was found that organisms grown at 27° in glucose broth were markedly more active than organisms grown at 37°. Investigation of the effect with the tyrosine decarboxylase of stock *Strep. faecalis* shows that the activity of the washed suspension does not vary significantly whether the culture is grown at 27 or 37°. To conform

with the previous work, however, all cultures were grown, unless otherwise stated, at 27°.

Age of culture. Table 1 shows the variations of *p*H medium, dry weight of organism in culture, and tyrosine decarboxylase activity of the washed suspension, with the "age of the culture" for stock *Strep. faecalis* growing in 2% glucose broth at 27°. The experimental details are the same as those previously described [Gale, 1940]. In other cases quoted, the organism was harvested between the 17th and 18th hr. of growth when its activity is maximal.

Table 1. *Variation of tyrosine decarboxylase activity with age of culture*

Hr. of growth	*p*H of medium	Dry wt. of organism in culture mg./ml.	Tyrosine decarboxylase Q_{CO_2}
8	7·5	0·005	103
10	7·2	0·01	143
12	7·0	0·055	286
14	6·5	0·275	325
16	6·1	0·350	330
18	5·7	0·420	286
20	5·6	0·470	243
30	5·4	0·490	180

p*H of growth medium.* It has been shown [Gale, 1940] that the decarboxylases of *Bact. coli* are formed most readily when the organism is grown in a medium of low *p*H. Thus when the organism is grown in a medium containing glucose, washed suspensions of high activity are obtained but the activity is due to the low *p*H produced in the medium during growth by fermentation acids. In order to investigate whether similar conditions hold with the tyrosine decarboxylase of *Strep. faecalis*, the stock organism was grown in flasks of plain broth, the *p*H having been previously adjusted in various cases to 8·0, 7·0, 6·0 and 5·0. These cultures were incubated at 27° and the organism harvested at the time, in each case, when the turbidity ceased to increase. *Strep. faecalis* does not grow readily in non-carbohydrate media but sufficient organism was obtained in each case for washed suspensions to be prepared and their tyrosine decarboxylase activities to be investigated at *p*H 5·0 and 30°. The results obtained are shown in Fig. 3 where it can be seen that this enzyme is formed by the organism in response to an acid growth environment, resembling in this respect the other decarboxylases that have been studied. The activity of the organism grown in glucose broth can thus be explained by the fall in *p*H during growth due to fermentation acids.

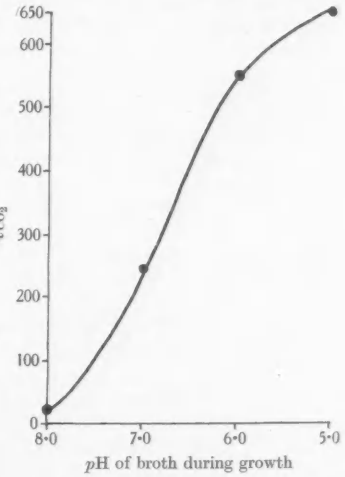

Fig. 3. Variation of tyrosine decarboxylase activity (Q_{CO_2}) of washed suspensions of stock *Strep. faecalis* with the *p*H of the plain broth in which the organism was grown.

Isolation and identification of tyramine as the dibenzoyl derivative

The amount of tyrosine used in the manometric experiments so far described was 0·5 ml. *M*/30 suspension. Decarboxylation of this to completion would liberate 373 μl. CO_2 and the total volume of gas evolved under the experimental conditions varied between 360 and 380 μl. Exact theoretical output cannot be expected as the amino-acid is used in suspension, but the results are sufficiently correct to show that the decarboxylation is quantitative. Isolation of the tyramine as the dibenzoyl compound is carried out as follows: 1 l. of 2 % glucose broth is inoculated with stock *Strep. faecalis* and incubated at 27° for 18 hr. The organism is then spun out of culture, washed on the centrifuge and made up in distilled water to a washed suspension of strength 10 mg./ml. 30 ml. of this suspension are then added to 30 ml. 0·05 *M* phthalate buffer *p*H 5·0 and 30 ml. *M*/60 tyrosine suspension in a Krebs's vessel. This is filled with N_2 and then incubated at 30° until a control manometric experiment indicates that the reaction has gone to completion (1·5–2 hr.). The organism is then removed by centrifuging and passage through a Seitz filter. The filtrate is acidified to about *p*H 1 and evaporated *in vacuo* to small bulk. The *p*H is then brought to 7·5–8 by the addition of solid $NaHCO_3$ and the mixture cooled in ice to 10–12°. Benzoyl chloride is then added with vigorous shaking, a few drops at a time until about 3 molecular equivalents have been added, the *p*H being maintained in the region of 8 by addition of $NaHCO_3$. The dibenzoyltyramine slowly precipitates out and the mixture is left overnight, when the precipitate is filtered off, washed with water and dried *in vacuo*. Twice recrystallized from abs. alcohol: M.P. 171–2° (corr.). Found: C, 76·4; H, 5·33; N, 4·03 % (Weiler). Calc.: C, 76·50; H, 5·54; N, 4·06 %. Yield 132 mg.

Decarboxylation of tyrosine by various strains of Strep. faecalis

The action of washed suspensions of other strains of *Strep. faecalis* has been tested under the usual experimental conditions on the 16 amino-acids listed above. The organisms were grown in 2 % glucose broth for 18 hr. at 27°; washed suspensions were prepared and their actions tested anaerobically at *p*H 5, etc. as indicated. In no case was any amino-acid, other than tyrosine, decarboxylated with the formation of an amine and in every case the action with tyrosine was optimal at *p*H 5·0. Arginine was attacked in some cases with the liberation of

Table 2. *Formation of tyramine and ornithine by* Strep. faecalis *grown in 2 % glucose broth*

Strain	Final *p*H in medium	Q_{CO_2} Tyrosine	Q_{CO_2} Arginine
1. Stock	4·95	243*	0
2. Grant A	4·84	221*	0
3. Grant B	5·38	68*	24
4. Sargent	5·70	14	61
5. Double	6·02	—	5
6. Hucker (N.T.C. 2703)	5·29	43	83
7. Stubbs (N.T.C. 370)	5·03	44*	17

* Tyramine isolated as dibenzoyl derivative and identified.

It can be seen that, generally speaking, the strains which attack tyrosine rapidly are those that produce acid rapidly in the growth medium.

2 mol. NH_3 and 1 mol. CO_2 per mol. arginine. This reaction has been studied by Hills [1939] for various *Streptococci* and the product is stated to be ornithine. Table 2 shows the Q_{CO_2} obtained with tyrosine at the optimum *p*H 5·0, the Q_{CO_2} obtained with arginine at *p*H 6·0 for the reaction arginine → ornithine + $(NH_4)_2CO_3$ and the *p*H attained in the medium on harvesting in representative cases.

Specificity of tyrosine decarboxylase

The strict specificity of this enzyme is emphasized by the list of amino-acids not attacked by any of the organisms decarboxylating tyrosine. Thus, as none of phenylalanine, tryptophan, alanine or serine is attacked, it would appear that the enzyme is specific for the complete *p*-hydroxyphenylalanine molecule. Although all the above tests were carried out anaerobically in the first place, the decarboxylation at *p*H 5·0 is not affected in any way by the presence of oxygen.

Variation of amino-acid constitution of growth medium

Table 3 gives the tyrosine decarboxylase activity (Q_{CO_2}) of *Strep. faecalis Grant A* grown in the following media:

Medium A: Stephenson's inorganic medium; 2% glucose; 0·1% marmite.
Medium B: as A + 0·3% tyrosine.
Medium C: acid hydrolysate of gelatin; 2% glucose; 0·1% marmite.
Medium D: tryptic digest of casein; 2% glucose.
Initial *p*H in all cases = 7·3.

Table 3. *Variation of tyrosine decarboxylase activity with constitution of growth medium* (Strep. faecalis Grant A)

Medium	Final *p*H	Q_{CO_2} (Tyrosine)
A	4·85	8
B	5·55	20
C	5·23	69
D	4·85	218

SUMMARY

1. Washed suspensions of stock *Strep. faecalis* decarboxylate *l*(−)-tyrosine to form tyramine.
2. The decarboxylase involved is optimally active at *p*H 5·0.
3. Cultures grown at 27° have the same activity as those grown at 37°.
4. The activity of the washed suspension varies with the age of the culture, maximum activity occurring between the 16th and 18th hr. when growth takes place in 2% glucose broth (tryptic digest of casein) at 27°.
5. The activity of the washed suspension depends upon the *p*H of the growth medium. Cultures grown in non-carbohydrate media at *p*H 7 have little activity, this being increased 30–40-fold by growth at *p*H 5. In carbohydrate media the activity obtained is explained by the fall in *p*H during growth due to fermentation acids.
6. The decarboxylation is quantitative and the tyramine has been isolated and identified as the dibenzoyl derivative.
7. Of 7 strains of *Strep. faecalis* investigated, 6 decarboxylated tyrosine. These active strains appear to be the more rapid acid-producing strains; 5 strains also formed ornithine by liberation of $(NH_4)_2CO_3$ from arginine.

8. In no case was any other amino-acid decarboxylated to form an amine under the experimental conditions described. The tyrosine decarboxylase is thus strictly specific.

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103. THE PRODUCTION OF AMINES BY BACTERIA

3. THE PRODUCTION OF PUTRESCINE FROM *l*(+)-ARGININE BY *BACTERIUM COLI* IN SYMBIOSIS WITH *STREPTOCOCCUS FAECALIS*

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(*Received 25 April 1940*)

IN the first paper of this series, the author [Gale, 1940, 1] showed that washed suspensions of *Bact. coli* acting at *p*H 4·0 carry out a simple decarboxylation of *l*(+)-arginine to produce agmatine. Previous work on amine production by bacteria growing in culture in the presence of arginine had given putrescine as the product [Ackermann, 1910; Hirai, 1936; Akasi, 1938; etc.]. It was suggested that this result was obtained, where mixed cultures were used as by Ackermann [1910], owing to the presence of organisms containing an "arginase" which produced ornithine from the arginine before decarboxylation occurred. Ackermann [1908] obtained ornithine from arginine in the presence of decomposing pancreas and the reaction has recently been obtained in the presence of washed suspensions of various streptococci by Hills [1939]. The reaction by *Strep. faecalis* has been confirmed by Gale [1940, 2] who obtained ornithine from arginine in the presence of 5 out of 7 strains tested. The enzyme responsible does not appear to be a true arginase as urea is not an intermediate product [Hills, 1939] and the actual course of the breakdown is obscure. An enzyme possessing the same properties has also been found in certain strains of *Lactobacillus acidophilus* by the author.

In the present communication the action of mixtures of *Bact. coli* and *Strep. faecalis* in washed suspension is discussed, the suspensions being obtained by artificial mixture of cultures grown separately and also from cultures grown in symbiosis.

Methods

The organisms were grown in a tryptic digest of casein to which glucose was added to a concentration of 2% and at a temperature of 27°. The preparation and investigation of activity of washed suspensions were essentially the same as described in the previous papers.

Organisms used

Bact. coli Esch. This organism has been shown [Gale, 1940, 1] to decarboxylate *l*(+)-arginine to agmatine at an optimum *p*H 4·0, *l*(+)-ornithine to putrescine at *p*H 5·0, *l*(+)-lysine to cadaverine at *p*H 4·5 and *l*(−)-histidine to histamine at *p*H 4·0.

Strep. faecalis Sargent. This organism produces ornithine from *l*(+)-arginine at an optimum *p*H 5·5 and also decarboxylates tyrosine to tyramine at a slow rate ($Q_{CO_2}=14$ at *p*H 5·0) [Gale, 1940, 2].

Strep. faecalis Grant A is a rapid tyramine producer ($Q_{CO_2}=221$) but does not attack arginine under the usual experimental conditions.

[1] Senior Student of the Royal Commission for the Exhibition of 1851.

Effect of artificial mixture of washed suspensions of Bact. coli Esch. *and* Strep. faecalis Sargent

Fig. 1 shows the variation with *p*H of (1) arginine decarboxylase activity of *Bact. coli*, (2) ornithine decarboxylase activity of *Bact. coli* and (3) "arginase" activity of *Strep. faecalis*. It can be seen that at *p*H 4·0 the arginine decarboxylase is optimally active whereas the other two enzymes are only slightly active, while at *p*H 5·5, the optimal value for the "arginase" enzyme, the arginine decarboxylase is almost inactive. It is to be expected that, if the properties of

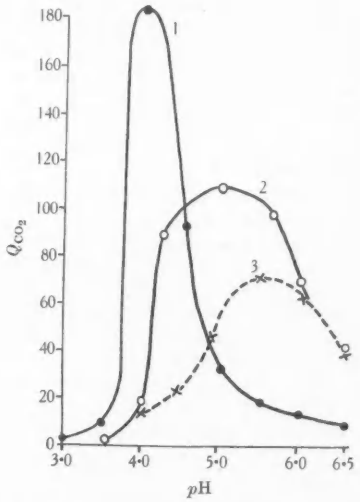


Fig. 1. Variation of activity (Q_{CO_2}) with *p*H of (1) arginine decarboxylase of *Bact. coli Esch.*, (2) ornithine decarboxylase of *Bact. coli Esch.*, (3) "arginase" of *Strep. faecalis Sargent.*

these enzymes remain unchanged by mixture of the organisms containing them, then the mixed culture should produce agmatine at *p*H 4·0 and putrescine at *p*H 5·5 from arginine, the latter reaction occurring with ornithine as intermediate product. The "arginase" of *Strep. faecalis* is inactive towards agmatine.

In the first place, washed suspensions of *Bact. coli Esch.* and of *Strep. faecalis Sargent* were prepared from cultures grown separately in 2 % glucose broth and their activities determined against the various amino-acids attacked. The suspensions were then mixed and these activities again determined as shown in Table 1.

From Table 1 it can be seen that in those cases where the substrate is attacked by only one of the organisms, the mixture of the organisms has no effect upon the rate of attack by the enzymes concerned. In the case of arginine at *p*H 5·5, the rate of attack by the mixed cultures is, however, greater than the sum of the rates of their separate attacks. This is due, presumably, to the fact that three reactions are occurring in this case:

1. Arginine → agmatine by the arginine decarboxylase of *Bact. coli.*
2. Arginine → ornithine by the "arginase" of *Strep. faecalis.*

3. Ornithine → putrescine by the ornithine decarboxylase of *Bact. coli* reacting with the ornithine formed in reaction 2. Thus the third reaction is made possible by the mixture of the organisms.

Table 1. *Decarboxylase activities of organisms before and after mixing*

Substrate	*p*H	*Bact. coli* mg./ml.	*Strep. faec.* mg./ml.	μl. CO_2 evolved per 10 min.
Ornithine	5·0	10·25	—	153
		—	13·5	0
		10·25	13·5	152
Lysine	4·5	3·4	—	140
		—	13·5	0
		3·4	13·5	128
Histidine	4·0	10·35	—	18
		—	13·5	0
		10·35	13·5	20
Tyrosine	5·0	10·25	—	0
		—	13·5	15
		10·25	13·5	15
Arginine	4·0	3·4	—	104
		—	13·5	3
		3·4	13·5	108
Arginine	5·5	10·3	—	48
		—	13·5	51
		10·3	13·5	141

The quantity of arginine used in the above experiment was 0·25 ml. of *M*/30 *l*(+)-arginine; thus if reaction (1) proceeds to completion, a total of 186 μl. CO_2 will be liberated, whereas if both reactions (2) and (3) go to completion, then 372 μl. CO_2 will be given off. From the rates of reaction given above, it appears that all three reactions occur simultaneously and the actual amount of gas evolved is 250–260 μl. At *p*H 4·0, when neither reaction 2 or 3 occurs at an appreciable rate, the total volume of CO_2 evolved in the presence of the mixed culture = 175 μl. The extent to which reaction 2 has taken place can be determined at the end of the experiment by estimation of the NH_3 liberated. The decarboxylases of *Bact. coli* are very thermolabile in washed suspensions and it is not to be expected that, under the experimental conditions used, all the ornithine formed by reaction 2 will be decarboxylated to putrescine. Calculations based upon the rates and quantities of CO_2 and NH_3 formation during the action of the pure and mixed cultures at *p*H 5·5 show that the products depend upon the ratio of the dry weights of *Bact. coli*/*Strep. faecalis* present; when this ratio = 1 the products appear to be agmatine, ornithine and putrescine in approximately equal amounts.

Action of a washed suspension of Bact. coli Esch. *and* Strep. faecalis Sargent *grown symbiotically*

1 l. 2% glucose broth was inoculated with approximately equal quantities of pure cultures of *Bact. coli Esch.* and *Strep. faecalis Sargent* and incubated at 27° for 18 hr.; the organisms were centrifuged off and made into washed suspension. At the same time a control suspension of *Bact. coli Esch.* alone was prepared. The activities (vol. CO_2 liberated/10 min.) of the symbiotic and pure cultures were determined against arginine, ornithine, lysine, histidine and tyrosine, the activity for arginine being determined at *p*H 4·0 and 5·5 while the other activities were determined at the known optimum *p*H values. In the case of the pure culture of *Bact. coli*, the dry weight of organism used was determined as usual

and the various Q_{CO_2} values calculated. Since the previous work has shown that mixing of the organisms does not affect the rate of attack of the enzymes, the dry weight of *Bact. coli* present in the symbiotic mixture can now be calculated. Table 2 shows an example of such an experiment:

Table 2. *Decarboxylating activities of symbiotic culture*

Substrate	*p*H	Q_{CO_2} *Bact. coli*	ml. symbiot. culture used	μl. CO_2/ 10 min.	Calc. dry wt. *coli*	Calc. dry wt. *coli*/ml. mixture
Lysine	4·5	230	0·25	109	2·83	11·32
Histidine	4·0	22·5	1·0	37	9·9	9·9
Arginine	4·0	179	0·25	82	2·74	10·96
Arginine	5·5	—	1·0	69	—	—
Ornithine	5·0	151	0·1	69	2·76	27·6
Tyrosine	5·0	0	1·0	71	—	—

From the activities with three of the substrates for which the mixing of the organisms has no effect—arginine at *p*H 4·0, lysine and histidine—the calculated amount of *Bact. coli* present/ml. of mixed culture = $10{\cdot}7 \pm 0{\cdot}8$ mg. However, the amount of *coli* present calculated from the ornithine activity is 27·6 mg., roughly 3 times as much. This figure is too high to agree with the turbidity measurements. It would thus appear that the symbiosis of *Strep. faecalis Sargent* with *Bact. coli Esch.* results in the ornithine decarboxylase activity of the latter organism being increased 250–300 %. This effect is presumably due to the production in the growth medium of ornithine by the action of the *Strep.* on arginine. In order to check this assumption, the experiment was repeated using stock *Strep. faecalis* (which contains no "arginase") in the place of *Sargent*; in this case, the calculations based upon the ornithine activity agreed with the others to within 7 %.

To obtain an idea of the relative numbers of the two organisms in the symbiotic culture, the washed suspension was serially diluted and plated out on (1) broth agar, (2) glucose-broth agar. After 24 hr. incubation, only *coli* colonies were visible on (1) while both types of colony could be counted on (2). In 3 cases, the ratios of *Bact. coli*/*Strep. faecalis* in suitable dilution were 127/109, 122/88, 52/40 respectively.

With regard to the reaction with arginine: at *p*H 4·0 the manometric results showed a simple decarboxylation unaccompanied by any significant NH_3 formation; at *p*H 5·5 the CO_2 and NH_3 produced indicated that the arginine had been mainly broken down through ornithine to putrescine and this proved to be the case upon isolation as will be shown. This more clean-cut result obtained with the symbiotic mixture as compared with the artificial mixture is presumably due to the effect of symbiosis upon the enzymes concerned.

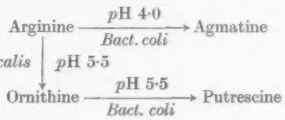
Isolation of the products of the action of the symbiotic culture on arginine

The organisms obtained from 1 l. of glucose broth as above were made up into 20 ml. washed suspension. Of this 10 ml. were mixed with 10 ml. 0·05 *M* phthalate buffer *p*H 4·0 and 10 ml. *M*/60 *l*(+)-arginine, and incubated anaerobically in a Krebs vessel. The remainder of the washed suspension was set up similarly but using buffer *p*H 5·5. The vessels were incubated at 30° for 2 hr. and the products then isolated as the picrates as previously described [Gale, 1940, 1]. The yields were not quantitative as in each case the product was contaminated with traces of either putrescine or agmatine but these traces could be removed by two or three recrystallizations.

The product obtained from the experiment at *p*H 4·0 gave orange needles, M.P. 238°; found (Weiler) N, 23·65 %: calc. for agmatine picrate, N, 23·81 %. Yield 56 mg.

The crystals obtained from the experiment at *p*H 5·5 appeared in the first place as yellow needles tinted red at the edges. Three recrystallizations from water gave bright yellow needles, M.P. 252° (decomp.); found (Weiler) N, 20·5 %; calc. for putrescine picrate, N, 20·44 %. Yield 73 mg.

Thus the action of a mixed culture of *Bact. coli Esch.* and *Strep. faecalis Sargent*, grown in symbiosis, can be represented diagrammatically as:



Summary

1. The mixture of washed suspensions of *Bact. coli Esch.* and *Strep. faecalis Sargent* has no effect upon the decarboxylation of *l*(+)-lysine, *l*(−)-histidine, *l*(+)-ornithine by the former or of *l*(−)-tyrosine by the latter. With *l*(+)-arginine, three reactions can occur: (1) decarboxylation of arginine to agmatine, (2) breakdown of arginine to ornithine, (3) decarboxylation of ornithine to putrescine. At *p*H 4·0, only the first reaction occurs to any extent; at *p*H 5·5, all three reactions occur, the ratio of the products depending upon the ratio of *Bact. coli*/*Strep. faecalis* present.

2. When these two organisms are grown in symbiosis, the activities of *Bact. coli* towards lysine, histidine and arginine at *p*H 4·0 appear to be the same as those of the pure culture. Towards ornithine, the activity increases 250–300 %; probably as a result of the production of ornithine during growth.

3. The organisms grown in symbiosis attack arginine at *p*H 4·0 to produce agmatine and at *p*H 5·5 to give putrescine.

The author wishes to express his thanks to Dr M. Stephenson and Prof. Sir F. Gowland Hopkins for their interest and encouragement in this work; also to the Royal Commissioners for the Exhibition of 1851 for the Senior Studentship during the tenure of which these investigations have been carried out.

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104. THE ESTIMATION OF PHOSPHORUS

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(*Received 26 April 1940*)

Osmond [1887] proposed the estimation of P by causing the formation of phosphomolybdic acid and its subsequent reduction to a blue compound which could be measured colorimetrically. Of the many variants of this procedure, that of Fiske & Subbarow [1925] has perhaps been the most widely adopted.[2] They carried out the reduction in strongly acid solution (0·5 *N* H_2SO_4) and used as reducing reagent a solution of 1-amino-2-naphthol-4-sulphonic acid in sulphite-bisulphite mixture. The principal advantages claimed for the method were (1) rapidity of colour development; (2) proportionality between colour density and amount of P over a wide range; (3) relative freedom from interference by such substances as ammonium and iron salts, nitrates, nitrites, silicates and chlorides.

King [1932] recommended the use of perchloric acid (final concentration 0·73 *N*) in place of sulphuric acid in the method of Fiske & Subbarow. This facilitates the preliminary destruction of organic material necessary for the determination of total P. The solubility of barium and calcium perchlorates confers added advantages, e.g. in determinations on plant extracts which contain sufficient calcium to cause troublesome precipitates with sulphuric acid and on barium and calcium salts of phosphoric esters.

The method of P estimation originally used by the author was a simple adaptation of King's perchloric acid method for use with an absolute photometer. An aliquot of the solution of orthophosphate[3] under examination was placed in a 25 ml. volumetric flask, and to it were added 2 ml. 60 % perchloric acid, 1 ml. 8·3 % ammonium molybdate and 1 ml. reducing reagent (0·417 g. 1-amino-2-naphthol-4-sulphonic acid, 5 g. crystalline sodium sulphite and 25 g. sodium bisulphite in 250 ml. water). Water was added to 25 ml. and after about 20 min. the extinction coefficient *K* (the extinction or colour density of a layer of solution 1 cm. thick) was measured in the photometer, using a deep red filter (Zeiss S 72) and an absorption cell of appropriate thickness (0·5–3 cm.). The concentration of P present was then read from a standardization (*K* against mg. P) curve established with known amounts of pure primary potassium phosphate.

The relation between *K* and the period of colour development with low, medium and high concentrations of P is shown in Table 1. A comparatively rapid increase in *K* is followed by a long period of slow increase. In order to avoid appreciable errors, it was thus necessary always to determine *K* after a

[1] Holder of Senior Research Award of the Department of Scientific and Industrial Research.

[2] The method of Lohmann & Jendrassik [1926], which is frequently cited in the German literature, differs only slightly from that of Fiske & Subbarow.

[3] The procedure for the estimation of total P differed only in that the material under examination was first digested with a slightly greater quantity (2·2 ml.) of perchloric acid (see p. 861).

definite period (20 min.), a matter of considerable inconvenience when a large number of analyses were to be made.[1]

A more serious objection became apparent when it was found that the extinction coefficient at 20 min. varies considerably with the temperature. This was shown in the following experiment.

Table 1. *Relation between time and extinction coefficient in King's method*

P, mg./25 ml. final solution ...	0·010	0·100	0·400
Av. temp. during exp. period ...	17°	14°	19°
Time	*K*	*K*	*K*
2 min.	0·060	0·503	1·540
5 ,,	0·060	0·530	1·960
8 ,,	0·061	0·535	2·044
20 ,,	0·063	0·550	2·145
30 ,,	0·064	0·558	2·180
50 ,,	0·069	0·574	2·200
3 hr.	0·074	0·635	2·370
7 ,,	0·082	0·730	2·600

Table 2. *Influence of temperature on extinction coefficient in King's method*

Av. temp. of solution during colour development (±1°)	*K* (20 min.)
−1·5°	0·370
+3·2°	0·530
8·0°	0·540
12·5°	0·548
16·6°	0·560
20·3°	0·568
24·3°	0·582
26·8°	0·588
34·4°	0·620

5 ml. standard phosphate solution (=0·100 mg. P) were placed in each of nine 25 ml. volumetric flasks and 2 ml. 60% perchloric acid and about 14 ml. water added to each. The flasks were brought to different temperatures, molybdate, sulphonic acid reagent and water to 25 ml. were added and the extinction coefficients determined after 20 min. (Table 2).

These features are less important when the solution under examination is always compared with a standard solution prepared simultaneously, but become serious when the analysis depends on the measurement of the absolute colour density. In the method of P determination described below these disadvantages are eliminated. The necessary period of colour development is also much reduced; not only is a considerable saving in time effected thereby, but the extent of hydrolysis of labile organic P compounds (e.g. glucose-1-phosphate) is proportionately reduced.

The modified method

Several references to the possibility of using amidol (2:4-diaminophenol hydrochloride) as the reducing agent in the colorimetric estimation of P are to be found in the literature. Fiske & Subbarow [1925] described amidol as superior to quinol for this purpose but (for reasons unstated) "not entirely satisfactory

[1] Brose & Jones [1936] have shown that in the case of the original Fiske & Subbarow [1925] method the colour density continues to increase for a number of hours.

from every standpoint," whilst Tschopp & Tschopp [1932] included it in a group of substances which were found satisfactory but rejected on economic grounds. Finally Müller [1935] found that if a solution of amidol in sodium sulphite is substituted for the sulphonic acid reagent in Fiske & Subbarow's method, the blue colour produced with amounts of P between 0·05 and 0·10 mg. reaches a maximum density within 5 min. and remains constant for a further 25 min. It has been found, using Müller's reagent in the presence of perchloric acid, that the colour produced tends to be purple and that the intensity fails to reach a steady maximum with amounts of P much above 0·1 mg. Further, Müller's reagent proved to be inconveniently unstable. These difficulties are eliminated in the procedure now described.

Solutions

Ammonium molybdate solution. An 8·3 % solution of the A.R. salt in distilled water. A small quantity of conc. ammonium hydroxide may be added to facilitate solution.

Perchloric acid. 60 % solution (specific gravity 1·54) A.R.

Amidol reagent. 2 g. amidol and 40 g. pure sodium bisulphite are dissolved in glass-distilled water and diluted to 200 ml. The reagent is best kept in a well-stoppered bottle painted black on the outside. It is advisable to discard the solution after about 10 days.

Hydrogen peroxide. 30 % ("100 volumes") solution, free from P. The "M.A.R." grade sold by Messrs British Drug Houses, Ltd. is suitable, but bottles in which the paraffin lining is broken should be rejected.

Standard phosphate solutions. A stock solution containing 1 mg. P per ml. is prepared by dissolving 1·0967 g. A.R. KH_2PO_4 (dried in an air oven) in distilled water and diluting to 250 ml. This solution remains unaltered for at least 2 years if stored over chloroform at 0°. Suitable standard solutions for the calibration of the photometer are obtained by dilution of the stock solution.

Estimation of orthophosphate

An aliquot ($\geqslant$ 20 ml.) of the solution under examination, containing up to 0·4 mg. P, is placed in a 25 ml. volumetric flask and to it are added 2 ml. perchloric acid, 2 ml. amidol reagent and 1 ml. ammonium molybdate in that order. Water to 25 ml. is added, the solution mixed and the extinction coefficient is determined after an interval of 5–30 min. The amount of P present is then read from a calibration curve (K against mg. P) obtained by applying the same procedure to aliquots of standard phosphate solutions containing from 0·01 to 0·4 mg. P.

Table 3 gives the calibration data for the deep red filter (Zeiss S 72) used in the present investigation.

Table 3. *Calibration data for the modified method, using a deep red filter (Zeiss S 72)*

P, mg./25 ml. final solution	K	mg. P/K
0·010	0·055	0·180
0·020	0·110	0·182
0·050	0·270	0·185
0·100	0·532	0·188
0·150	0·798	0·190
0·200	1·036	0·193
0·300	1·564	0·192
0·400	2·070	0·193

Estimation of total P

An appropriate amount of the material (solid or solution) under examination is placed in a micro-Kjeldahl flask (hard glass) and 2·2 ml.[1] perchloric acid and a small piece of porous pot (to prevent bumping) are added. The flask is heated over a micro-burner until the contents have become colourless [King, 1932]. The addition of a few drops of H_2O_2 may be required to complete the combustion if much organic material is present. After cooling the flask, its contents are thoroughly rinsed into a 25 ml. volumetric flask, 2 ml. amidol, 1 ml. molybdate and water to 25 ml. are added, and the extinction coefficient is determined between 5 and 30 min. later.

If the highest accuracy is not essential, considerable time may be saved by developing the blue colour in the digestion flask. In order to do this 20 ml. distilled water, followed by 2 ml. amidol and 1 ml. molybdate, are added to the cooled colourless digest and the extinction coefficient is determined as before. This abbreviated procedure will involve no important error as long as the volume of perchloric acid at the end of the digestion does not differ appreciably from 2 ml. It has been employed successfully for some time with trichloroacetic acid extracts of apples and potatoes; the following experiment (Table 4) shows that even in the presence of large amounts of organic material (and NaCl) a good recovery of added P is obtained.

Table 4

0·100 mg. P (as KH_2PO_4) was added to each of 10 digestion flasks together with 2·2 ml. perchloric acid, a small piece of porous pot and 0·5 g. of the substance indicated. The digestion and colour development were then carried out by the abbreviated method.

No.	Substance added	mg. P recovered
1	Glucose	0·100
2	Glucose	0·100
3	Glycine	0·100
4	Glycine	0·100
5	Oxalic acid	0·100
6	Oxalic acid	0·101
7	Citric acid	0·100
8	Ammonium acetate	0·101
9	Sodium chloride	0·103
10	Sodium chloride	0·103

The only substance so far encountered which is not readily digested by the King perchloric acid technique is urea. If a considerable amount of this substance is present the contents of the digestion flask become solid, and if the heating is prolonged most of the perchloric acid is volatilized. According to Rae & Eastcott [1939] this difficulty may readily be overcome by a preliminary treatment of the urea solution with HNO_3.

Relation between extinction coefficient and time

The extinction coefficients with 0·010, 0·100 and 0·400 mg. P observed at different times after addition of the usual reagents are shown in Table 5. It will be seen that during the interval between 3 and 30 min. the colour density in each case was virtually constant. Later a slow increase is detectable.

[1] This slightly larger amount of perchloric acid is used in order to compensate for the loss (0·2 ml.) during digestion [King, 1932].

Table 5. *Relation between time and extinction coefficient in the modified method*

P, mg./25 ml. final solution ...	0·010	0·100	0·400
Time after addition of molybdate		*K*	
1 min.	0·049	0·498	—
1·5 „	0·055	—	—
2 „	—	0·525	—
3 „	—	0·531	2·060
4 „	—	—	2·070
15 „	0·055	0·531	2·070
30 „	0·055	0·531	2·070
40 „	0·056	0·532	2·100
60 „	0·057	0·535	2·130
2 hr.	0·059	0·548	2·220
3·7 „	0·066	0·590	—
6·5 „	0·076	0·635	—

Influence of temperature on extinction coefficient

The results in Table 6 were obtained in an experiment analogous to that summarized in Table 2, but using the modified method of P estimation. The extinction coefficient was measured after 5 and after 30 min. colour development at the temperatures indicated.

Table 6. *Influence of temperature on extinction coefficient in the modified method*

Av. temp. during the period 0–30 min. (±1°)	P, mg./25 ml. final solution	*K* (5 min.)	*K* (30 min.)
8·2	0·010	0·055	0·055
10·4	0·010	0·055	0·055
17·1	0·010	0·055	0·055
26·0	0·010	0·055	0·055
27·8	0·010	0·055	0·057
32·8	0·010	0·068	—
12·0	0·100	0·532	0·532
16·0	0·100	0·531	0·532
19·6	0·100	0·532	0·532
24·5	0·100	0·532	0·533
8·0	0·400	1·980	2·070
8·2	0·400	2·070	—
27·9	0·400	2·070	2·080
28·4	0·400	2·070	2·110
33·5	0·400	2·070	2·200

It will be observed that variations in temperature between approximately 8° and 26° do not affect significantly the extinction coefficient during the 5–30 min. interval.

Effect of variation of the quantities of perchloric acid, amidol reagent and ammonium molybdate used in the estimation

From Table 7 it will be seen that the amount of perchloric acid per 25 ml. of final solution may vary between 1·0 and 2·4 ml. without sensibly affecting the extinction coefficient of the solution; the amount of amidol reagent may vary

between 1·7 and 2·2 ml., and that of molybdate between 0·9 and 1·5 ml. (1·1 ml. in the case of very low amounts of P).[1]

Table 7. *Effect of variation of the quantities of reagents used*

Perchloric acid			Amidol reagent			Ammonium molybdate		
	Amount added	K		Amount added	K		Amount added	K
mg. P	ml.	(5–30 min.)	mg. P	ml.	(5–30 min.)	mg. P	ml.	(5–30 min.)
0·100	1·0	0·532	0·010	1·8	0·055	0·010	0·9	0·055
0·100	1·5	0·533	0·010	**2·0**	**0·055**	**0·010**	**1·0**	**0·055**
0·100	1·9	0·531	0·010	2·2	0·055	0·010	1·1	0·055
0·100	**2·0**	**0·532**				0·010	1·2	0·060
0·100	2·2	0·532	0·400	1·7	2·065	0·010	1·3	0·064
0·100	2·3	0·532	0·400	1·8	2·065			
0·100	2·4	0·532	0·400	**2·0**	**2·070**	0·400	0·7	2·000
0·100	2·5	0·500	0·400	2·2	2·080	0·400	0·8	2·050
0·100	3·0	0·461	0·400	2·3	2·096	0·400	0·9	2·070
						0·400	**1·0**	**2·070**
						0·400	1·3	2·080
						0·400	1·5	2·076

Effect of interfering substances

In Table 8 are given the approximate maximum concentrations of a number of substances which may be present in the final solution in the estimation of P without interfering with the analysis. The first 8 substances when present in concentrations higher than those indicated cause a decrease in colour density, the last 2 an increase.

Table 8. *Maximum permissible concentrations of interfering substances*

		Permissible concentration in presence of		
		0·400 mg. P	0·010 mg. P	
No.	Substance	M	M	Remarks
1	$(NH_4)_2SO_4$	0·4	0·75	—
2	NaCl	0·5	0·6	—
3	$NaNO_3$	0·5	0·8	—
4	$CCl_3.COOH$	0·25	0·5	—
5	NaF	0·01	0·02	—
6	$K_2C_2O_4, H_2O$	0·002	0·004	—
7	$FeCl_3$	0·0013	0·0013	—
8	Na_2SiO_3	0·0043*	0·00011*	Ratio Si : P = 7·5
9	C_2H_5OH	1·8	1·8	Equivalent to 2·5 ml. 95 % alcohol per 25 ml. final solution

* The sensitivity to silicate is considerably greater if the molybdate is added to the test solution before the amidol reagent [cf. Tschopp & Tschopp, 1932].

The effects of most of the above substances on the determination of P by the method of Fiske & Subbarow [1925] were examined by these authors and by Vársárhelyi [1930]; King [1932] made a shorter but similar study in connexion with his modified method. Although the maximum permissible concentrations of these substances were not clearly defined in these investigations, it can be stated that the present method exhibits at least as high a degree of tolerance as the older methods.

[1] It is, of course, not suggested that the reagents be added so carelessly as to give rise to variations of this magnitude. In the presence of interfering substances the tolerances to variations in the amounts of reagents used may be considerably less than these indicated here.

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A method for the determination of phosphorus in turbid or highly coloured solutions

The determination of orthophosphate in higher plant extracts is frequently complicated by the fact that these exhibit appreciable turbidity or colour; this is the case, for example, with trichloroacetic acid extracts of potatoes, apples, bananas and onions. The turbidity, due apparently to the presence of polysaccharide material, increases in some cases after the addition of perchloric acid and molybdate. An approximate correction for these effects may be applied if a separate measurement is made of the extinction value exhibited by a blank determination from which the reducing reagent is omitted; this correction cannot be made, however, when the extract contains strong reducing substances, e.g. ascorbic acid.

To overcome a somewhat similar difficulty encountered with extracts of animal tissue Delory [1938] has recommended the preliminary precipitation of orthophosphate by the addition of $CaCl_2$, NH_4OH and "light" magnesium carbonate; the precipitate is separated on the centrifuge, and, after washing, the determination is carried out on this material. When this process is applied to extracts of higher plants, however, only a small proportion of the inorganic phosphate present is precipitated. Thus with trichloroacetic acid extracts of the banana, only a trace of inorganic phosphate is precipitated, and even if a double excess of the precipitating reagents is employed and the time allowed for precipitation is increased up to 4 hr., only about a quarter of the phosphate present is thrown down. The Delory procedure is therefore not generally applicable to extracts of the higher plants.

It was hoped that the difficulty might be eliminated by the use of the Berenblum & Chain [1938] modification of the Kuttner & Cohen [1927] method. In this, H_2SO_4 and molybdate are added to the solution of orthophosphate to be analysed and the yellow molybdic acid is extracted from the aqueous phase with *iso*butyl alcohol. The alcohol layer is removed and washed, shaken with stannous chloride solution for a standard time and the blue colour, which remains in the *iso*butyl alcohol, measured in a photometer. When the method is applied to trichloroacetic acid extracts of bananas, however, two defects become apparent: (1) added inorganic phosphate can only be recovered to the extent of 70–80 %; (2) when *iso*butyl alcohol is shaken with a trichloroacetic acid extract without previous addition of molybdate, some material passes into the alcohol layer which gives a distinct blue colour with the subsequent reagent.

While the method of Berenblum & Chain [1938] is thus not applicable to extracts from certain higher plants at least, the following procedure, based on extraction of the reduced phosphomolybdic acid in *iso*butyl alcohol, has been used successfully.

A peculiar defect observed in the first trials of the method was that the density of the blue colour increased at an appreciable rate after its extraction into *iso*butyl alcohol. This effect, which was apparently due to reduction of molybdic acid, has been eliminated by the addition to the aqueous solution, before the extraction, of enough oxalic acid to combine with the excess molybdate. (Molybdic and oxalic acids combine to form a very stable oxalomolybdic acid [see Davies & Davies, 1932].) The extracted blue colour is thereby rendered stable for at least 24 hr.

Solutions. Besides those described on p. 860, ethyl alcohol (95 %), *iso*butyl alcohol (redistilled) and 10 % (saturated) oxalic acid solution are required.

Procedure. An aliquot ($\geqslant$ 20 ml.) of the solution of orthophosphate under examination, containing up to 0·4 mg. P, is placed in a 25 ml. volumetric flask

and treated exactly as described under "Estimation of orthophosphate" on p. 860. After the blue colour has developed for from 5 to 30 min., 1 ml. 10% oxalic acid is added. After mixing thoroughly, the contents of the flask are transferred quantitatively to a separating funnel, the flask being rinsed out with a little *iso*butyl alcohol. About 10 ml. *iso*butyl alcohol are then added to the contents of the funnel, the mixture is swirled for a few moments, the phases are allowed to separate and the aqueous (lower) layer is run off into a clean beaker. The blue *iso*butyl alcohol layer is run into a 25 ml. flask and the sides of the funnel are rinsed into the same flask with a little ethyl alcohol. The aqueous phase is then returned to the funnel and the extraction repeated with a further 5 ml. of *iso*butyl alcohol. The combined *iso*butyl alcohol extracts are then diluted to 25 ml. with ethyl alcohol, and the extinction coefficient is measured within 24 hr. The amount of P present is read from a calibration curve (K against mg. P) obtained by applying the same procedure to a series of standard phosphate solutions.

The calibration data for the deep red filter used in the present investigation are given in Table 9.

Table 9. *Calibration data for the* iso*butyl alcohol extraction method, using a deep red filter (Zeiss S 72)*

mg. P	K	mg. P/K
0·010	0·064	0·156
0·050	0·319	0·157
0·080	0·505	0·158
0·100	0·631	0·158
0·125	0·760	0·164
0·150	0·920	0·163
0·200	1·220	0·164
0·300	1·820	0·165
0·400	2·440	0·164

SUMMARY

Certain defects in the King-Fiske & Subbarow method of P estimation, which become serious when the method of analysis depends on the determination of absolute colour density, are described. A method of P estimation is proposed in which these adverse features are eliminated.

A procedure for the determination of P in cloudy or highly coloured solutions is described.

The author's thanks are due to Dr C. S. Hanes for permission to include some results obtained by him with *iso*butyl alcohol.

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105. THE ACTION OF SULPHUR-CONTAINING AMINO-ACIDS AND PROTEINS ON LIVER FAT DEPOSITION

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An investigation of the effect of pure amino-acids on the degree of fatty infiltration into the livers of rats maintained on high-fat, choline-free diets was a natural extension of the observation of the lipotropic action of proteins [Channon & Wilkinson, 1935; Best *et al.* 1936; Channon, Loach *et al.* 1938]. The discovery of the effect of cystine in causing marked acceleration of liver fat deposition [Beeston & Channon, 1936; Tucker & Eckstein, 1937] and of the contrasting finding that methionine prevented fat deposition to a considerable extent [Tucker & Eckstein, 1937; Channon, Manifold & Platt, 1938, 1; Best & Ridout, 1940] were the firstfruits of this method of approach. At the present time nothing is known regarding the means whereby these two amino-acids exert their opposing effects on fat deposition; in the case of methionine, however, it may be assumed as a working hypothesis that this amino-acid is involved in choline synthesis. In this connexion, interesting observations have been made by du Vigneaud *et al.* [1939], who found that supplements of homocystine were without effect on the growth of young rats receiving a diet deficient in methionine, but that the further addition of choline resulted in good growth; in contrast, the addition of triethylcholine had no effect and, since Channon & Smith [1936] found this latter compound to possess lipotropic activity, it must be concluded that the action of choline in these growth experiments is not to be associated with its effect in preventing liver fat deposition. These results suggest that choline can supply methyl groups for the conversion of homocysteine into methionine for growth-promoting purposes. Du Vigneaud *et al.* made the further tentative suggestion that the action of methionine on liver fat might be due to its provision of methyl groups for choline synthesis. Such a suggestion is not improbable, for on present evidence the demethylation of methionine to homocysteine is a likely initial step in the catabolism of this amino-acid. In a previous paper, an endeavour was made to make a quantitative estimate of the action of methionine in terms of choline [Channon, Manifold & Platt, 1938, 1], and the present paper records the results of further studies designed to throw light on its mode of action; evidence on this question has also been sought by the use of *S*-methylcysteine and homocystine. In the case of the contrasting action of cystine in increasing fat deposition, no simple theory suggests itself, and preliminary experiments on the level of blood fatty acids in animals receiving high fat diets with and without cystine were carried out to see if the accelerating action of cystine on liver fat deposition could be correlated with blood fat changes.

Since methionine exerts a preventive action on fat deposition in the liver, the further question arises as to whether the action of proteins in this respect is

solely a function of their methionine contents. This question is not easy to study because, as already pointed out, one at least of the amino-acids contained in proteins, namely cystine, markedly increases fat deposition. The behaviour of any protein in affecting fat deposition might therefore be due to the net result of the actions of these two amino-acids, a suggestion first made by Tucker & Eckstein [1937]. Study of the cystine and methionine contents of proteins in relation to their lipotropic actions will not confirm the accuracy of this suggestion unless quantitative information is available concerning both the relationship of the amount of each amino-acid ingested to the effect on liver fat and the effect of adding mixtures of the two amino-acids to fatty-liver diets. Experiments to be reported here, in which varying amounts of cystine and methionine were added to fatty-liver-producing diets, were designed to throw light on this point.

Experimental

In all the experiments groups of 10 rats of 150–200 g. weight were used; the groups had the same average body weight at the commencement and in the different experiments contained animals either of one sex or of the two sexes evenly divided. Experiments were carried out both on the "fat" and on the "cholesterol" fatty liver. The basal diets used in producing the "fat" fatty liver followed the general principles laid down by Channon & Wilkinson [1935] and were of the type: caseinogen 8, beef dripping 40, glucose 46, salt mixture 5, cod liver oil 1 part and aneurin 10 μg. per rat per day. This diet varied from experiment to experiment only in the proportion and nature of the protein, the glucose being varied at the expense of the protein. In discussing the separate experiments we shall refer to the diets by their protein contents, e.g. 8% egg albumin diet. In Exp. 1 the "cholesterol" fatty liver was studied, the diet being caseinogen 8, beef dripping 20, glucose 64, salt mixture 5, cholesterol 2, cod liver oil 1 part with 10 μg. aneurin per rat per day.

Except in Exp. 1 the individual livers were analysed by the usual saponification method, while in the "cholesterol" fatty liver study, pooled minced livers were extracted with alcohol and ether and the ether-soluble material analysed [Channon, Manifold & Platt, 1938, 1]. Total blood fatty acids were determined by the method of Stoddart & Drury [1929] as modified by Stewart & Hendry [1935]; blood samples were collected by severing the main neck vessels after stunning the animals.

dl-S-*Methylcysteine* was prepared, according to du Vigneaud *et al.* [1934]. (Found: C, 35·14; H, 6·62; N, 10·50; S, 2·38%. $C_3H_7O_2NS$ requires C, 35·50; H, 6·52; N, 10·44; S, 2·39%.) The formyl derivative melted at 119°.

dl-*Homocystine* was a sample kindly given to us by Dr du Vigneaud.

Determinations of the cystine and methionine contents of the proteins used were carried out by the method of Baernstein [1936].

Results

Liver fat

In previous work [Channon, Manifold & Platt, 1938, 1], it was found that a daily intake of 40 mg. methionine per rat had no effect in preventing fat deposition on diets which resulted in liver fat of 15%; in contrast, amounts of this order were effective when control diets resulting in a liver fat content of 20–25% were used. The object of Exp. 1 was to study the effects of smaller intakes of methionine and, for the reason just given, the diet was designed to

cause very fatty livers. The use of a basal diet supplemented with cystine fulfilled this condition and also provided information as to the effect of adding mixtures of cystine and methionine to a low-protein diet. It was also important to confirm the observation of Beeston & Channon [1936] that there was no relation between the amount of cystine fed and the liver fat resulting (intakes of 7–80 mg. per rat per day were studied). Clearly a definite knowledge of the effect of small amounts of cystine is essential for complete understanding of the part played by dietary protein in the control of liver fat.

Accordingly, 13 groups of rats were maintained on the cholesterol diet. One group served as control, five received supplements of 0·05, 0·10, 0·15, 0·20, 0·25 % cystine, four received 0·25 % cystine and 0·20, 0·30, 0·40, 0·50 % methionine and three groups received 0·25 % cystine and 0·01, 0·02, 0·03 % choline. The last three groups were included with a view to assaying the effects of smaller amounts of methionine than previously used in terms of choline. All the experimental data are collected in Table 1.

Table 1. *The effects of cystine and methionine in fatty liver production*

Exp. 1. Period of feeding 19 days

Group		Liver wt. as % of final body wt.	Cystine content of the diet %	Methionine content of the diet %	Total fatty acids: % fresh liver wt.	Total fatty acids: g. in liver of 100 g. rat
1	Control	5·34	0·04	0·22	21·70	1·17
2	+0·05 % cystine	6·05	0·09	0·22	27·10	1·63
3	+0·10 % cystine	6·09	0·14	0·22	28·30	1·72
4	+0·15 % cystine	6·24	0·19	0·22	27·22	1·75
5	+0·20 % cystine	6·44	0·24	0·22	26·20	1·69
6	+0·25 % cystine	6·16	0·29	0·22	25·85	1·61
7	+0·25 % cystine +0·20 % methionine	4·47	0·29	0·42	13·92	0·62
8	+0·25 % cystine +0·30 % methionine	4·29	0·29	0·52	13·68	0·59
9	+0·25 % cystine +0·40 % methionine	4·64	0·29	0·62	13·53	0·61
10	+0·25 % cystine +0·50 % methionine	4·22	0·29	0·72	13·14	0·56
11	+0·25 % cystine +0·01 % choline	5·57	0·29	0·22	25·43	1·42
12	+0·25 % cystine +0·02 % choline	4·94	0·29	0·22	18·67	0·95
13	+0·25 % cystine +0·03 % choline	5·32	0·29	0·22	20·53	1·09

The five concentrations of cystine administered have all produced the same increased degree of liver fat deposition, the figures varying only from 1·61 to 1·75 g. of fatty acids in the liver of the 100 g. rat. Thus 0·25 % added cystine has had no greater effect than 0·05 %, which corresponds to an intake of 4 mg. extra cystine per day. In confirmation of previous findings, these small supplements of cystine have increased liver fat by some 50 % over its already very high value. As with the cystine groups, the liver fat values of the animals receiving both cystine and increasing amounts of methionine show no differences among themselves. Since the average figure for liver fat of the methionine groups, 0·59 g., is not only considerably less than that for the group receiving cystine alone, 1·68 g., but also no more than one-half that of the control group, 1·17 g., it is clear that a daily extra intake of 16–40 mg. methionine not only inhibits the effect of cystine but even reduces the liver fat much below the control value. In group 11, a daily intake of about 0·8 mg. choline has resulted in the weight of liver fatty acids, 1·42 g., differing but little from the corresponding group 6. In groups 12 and 13 which probably ingested 1·6 and 2·4 mg. choline daily, the weights of liver fatty acids are 0·95 and 1·09 g.; these variations are insignificant and the failure to obtain different effects from doses differing so

little as 2·4 and 1·6 mg. is not unexpected. Making the reasonable assumption that 2 mg. choline have restored the liver fat to the control figure of 1·17 g., then 2 mg. choline have prevented the cystine action. From the results of this experiment, it is not possible however to make any certain estimate of the activity of methionine in terms of choline.

Table 2. *Analysis of liver lipins of Exp. 1*

		% of fresh liver			Wt. in liver of 100 g. rat		
Group		Free cholesterol	Cholesterol oleate	Glyceride	Free cholesterol	Cholesterol oleate	Glyceride
1	Control	0·49	4·86	20·44	0·03	0·26	1·09
2–6	+ cystine	0·32	4·25	26·22	0·02	0·26	1·63
7–10	+ cystine + methionine	0·31	3·28	12·71	0·01	0·14	0·56
11	+ 0·25 % cystine + 0·01 % choline	0·34	4·34	24·61	0·02	0·24	1·37
12	+ 0·25 % cystine + 0·02 % choline	0·37	4·26	18·40	0·02	0·21	0·92
13	+ 0·25 % cystine + 0·03 % choline	0·33	4·09	19·60	0·02	0·22	1·04

In order to obtain a more complete picture of these effects, detailed analyses were carried out on the control, choline, pooled cystine and pooled methionine groups. The phosphatide figures are omitted from the table since they showed no variations; the results for the glyceride content reflect the figures for total fatty acids which have already been discussed; the free and esterified cholesterol figures are, however, of interest. The control, cystine and cystine + methionine groups gave 0·03, 0·02 and 0·01 g. free cholesterol and 0·26, 0·26 and 0·14 g. cholesteryl oleate respectively in the 100 g. rat liver. These figures confirm our previous observation that the administration of methionine with cholesterol-containing diets markedly decreases the deposition of cholesterol, both free and esterified; cystine, on the other hand, exerts no effect on cholesterol [Channon, Manifold & Platt, 1938, 1].

In Exp. 2, the effects of still lower methionine dosages were studied; this experiment was similar in plan to Exp. 1 except that the groups receiving graded cystine supplements were omitted. Nine groups of rats were maintained on an 8 % caseinogen diet, one serving as control, and the other eight receiving 0·20 % cystine. In addition, three of these groups received 0·01, 0·02, 0·03 % choline respectively, and another four 0·05, 0·10, 0·15, 0·20 % methionine. All the results are recorded in Table 3.

Table 3. *Effects of choline and methionine on the "cystine" fatty liver*

Exp. 2. Period of feeding 17 days

		Intake of supplements in mg. per rat per day			Liver wt. as % of final body wt.	"Fat" in fresh liver %	"Fat" in liver of 100 g. rat g.
Group		Cystine	Methionine	Choline			
1	Control	—	—	—	5·02	24·60	1·23
2	+ 0·20 % cystine	16·6	—	—	6·44	29·80	1·91
3	+ 0·20 % cystine + 0·01 % choline	17·6	—	0·9	6·02	25·45	1·53
4	+ 0·20 % cystine + 0·02 % choline	18·0	—	1·8	5·20	25·20	1·31
5	+ 0·20 % cystine + 0·03 % choline	15·4	—	2·3	5·35	25·30	1·35
6	+ 0·20 % cystine + 0·05 % methionine	17·2	4·3	—	5·48	29·50	1·62
7	+ 0·20 % cystine + 0·10 % methionine	16·6	8·3	—	5·48	28·11	1·54
8	+ 0·20 % cystine + 0·15 % methionine	17·6	13·2	—	4·80	21·47	1·03
9	+ 0·20 % cystine + 0·20 % methionine	16·8	16·8	—	4·87	22·28	1·08

The addition of 0·20 % cystine to the basal diet has resulted in an increase of some 5·6 % in liver fat as compared with the control value. A daily intake of only 0·9 mg. choline per rat (group 3) has brought about some decrease in the extra fat deposition caused by cystine, while 1·8 or 2·3 mg. choline (groups 4 and 5) has virtually overcome the effect of cystine, for the values do not differ significantly from the control figure of group 1. This confirms the finding of the previous experiment, that 0·02–0·03 % choline is sufficient to prevent the increased deposition of liver fat caused by cystine; as in Exp. 1 no difference is seen in the effects of 0·02 and 0·03 % choline. In the groups which received both cystine and methionine, daily extra intakes of 4·3 and 8·3 mg. methionine (groups 6 and 7) have both exerted some preventive action while extra intakes of 13·2 and 16·8 mg. (groups 8 and 9) not only prevent the increase due to cystine but also lower the liver fat as compared with the control group. One important point is that the livers of the groups receiving 0·15 and 0·20 % methionine contain the same amounts of fat; 21·47 % and 1·03 g., 22·28 % and 1·08 g. respectively; in the previous experiment groups receiving supplements of methionine ranging from 0·20 to 0·50 % also gave values not differing from each other. The conclusion to be drawn is that under these conditions the lipotropic action of methionine is independent of the dose, provided that the dose is greater than a certain minimum value.

The results of this experiment enable a reasonably accurate comparison of the relative effects of methionine and choline to be made. Taking as standard the amount of either substance required to prevent the action of 0·20 % cystine, it appears that about 10 mg. methionine are equal in action on the extra deposition of liver fat caused by cystine to about 2 mg. choline.

In Exps. 1 and 2, the inclusion of 0·02 and 0·03 % choline was found to overcome the effects of cystine. Since the results in neither case differentiated between the effects of these two dosages, it seemed of value to attempt to define the effective dose more clearly. The results of Exp. 3 in which 9 % caseinogen was used in the diet, are presented in Table 4.

Table 4. *Effect of choline on the "cystine" fatty liver*

Exp. 3. Period of feeding 16 days

Group		Choline intake mg./rat/day	Liver wt. as % of final body wt.	"Fat" as % of wet liver wt.	"Fat" in liver of the 100 g. rat, g.
1	Control	—	4·47	17·34	0·80
2	Control	—	4·26	18·48	0·83
3	+0·25 % cystine	—	5·58	24·23	1·55
4	+0·25 % cystine +0·01 % choline	0·90	4·87	20·11	1·01
5	+0·25 % cystine +0·02 % choline	1·88	4·59	16·24	0·78
6	+0·25 % cystine +0·03 % choline	2·74	4·05	10·05	0·43

In this experiment the addition of 0·25 % cystine has caused a 90 % increase in liver fat; this increase is completely prevented by 1·9 mg. choline per rat per day: a further increase in choline intake to 2·7 mg. has resulted in a profound decrease of liver fat to 10·05 % and 0·43 g. The finding that 1·9 mg. choline are the effective daily intake of choline necessary to overcome the effects of cystine thus confirms the figure of 2 mg. which was deduced from the results of Exps. 1 and 2. Thus, in the prevention of the effects of cystine, choline is five times more effective than methionine on a weight basis.

To correlate these observations of the effect of mixtures of cystine and methionine on liver fat deposition with those of the lipotropic action of proteins, the effect of adding cystine to diets of increasing caseinogen content was studied. Four groups of animals received a 40% fat diet containing 8, 12, 15 and 25% caseinogen respectively while four more groups received the same diets supplemented by 0·25% cystine. The results are presented in Table 5.

Table 5. *The effect of caseinogen on the "cystine" fatty liver*

Exp. 4. Period of feeding 19 days

Group	Caseinogen content of diet %	Dietary content of cystine and methionine, derived from caseinogen %		Added cystine %	"Fat" % fresh liver	"Fat" in liver of 100 g. rat g.
		Methionine	Cystine			
1*a*	8	0·22	0·04	—	21·1	1·00
1*b*	—	—	—	0·25	29·2	1·66
2*a*	12	0·33	0·06	—	12·0	0·53
2*b*	—	—	—	0·25	21·7	1·04
3*a*	15	0·41	0·075	—	12·7	0·52
3*b*	—	—	—	0·25	16·2	0·72
4*a*	25	0·68	0·125	—	7·6	0·29
4*b*	—	—	—	0·25	8·1	0·30

The results of groups 1*a*–4*a* illustrate the now well-established lipotropic action of caseinogen; greater importance however lies in the fact that the addition of cystine results in an increased fat deposition at all but the highest protein intakes. Thus in group 1*b* cystine has caused a 66% increase in the already high fat content of the liver, in group 2*b* the increase approaches 100%, in group 3*b* it is 40% while in group 4*b*, in which the protein content of the diet is 25%, the cystine action disappears.

All the experiments so far described have been carried out with caseinogen as the dietary protein. Channon, Loach *et al.* [1938] showed that when egg albumin was substituted for caseinogen in low protein diets, the livers became much more fatty, and they suggested that possibly the greater cystine content of albumin might be responsible for the behaviour of this protein. In order to obtain evidence on this question, two experiments were carried out to see whether the addition of cystine to diets containing egg albumin would increase the deposition of liver fat. The relevant data of these experiments are given in Table 6, and all that needs to be added is that in Exp. 6 an 8% albumin diet was used while in Exp. 7 the diet contained 10% albumin.

Table 6. *Effect of supplementing an albumin diet with cystine*

Exp.	Period of feeding days	Group	Dietary content of cystine and methionine %		Liver % body wt.	"Fat" % fresh liver wt.	"Fat" in liver of 100 g. rat
			Methionine	Cystine			
6	17	1. Control	0·31	0·20	5·5	25·23	1·43
		2. +0·25% cystine	0·31	0·45	5·4	26·55	1·52
7	18	3. Control	0·39	0·25	5·8	23·90	1·47
		4. +0·5% cystine	0·39	0·75	6·1	25·13	1·61

Clearly, cystine does not exert its action on liver fat deposition when egg albumin is the dietary protein, for in neither experiment has the addition of

cystine resulted in amounts of liver fat differing significantly from the control figures. This may indicate that the very fatty livers resulting from albumin diets are a result of their high cystine contents—in these two experiments 0·20 and 0·25 % respectively.

Finally, in Table 7 are presented the results of experiments with *S*-methylcysteine, taurine and homocystine, a preliminary report of which has already been published [Channon, Manifold & Platt, 1938, 2]. The diets used were as follows: Exp. 8, 5% caseinogen; Exp. 9, 8% egg albumin; Exp. 10, 8% caseinogen.

Table 7. *Effects of* S-*methylcysteine, homocystine and taurine on fatty liver production*

Exp.	Period of feeding days	Group	Liver % body wt.	"Fat" % fresh liver wt.	"Fat" in liver of 100 g. rat
8	11	1. Control	4·1	16·36	0·68
		2. +0·28 % *S*-methylcysteine	4·5	16·48	0·79
		3. +0·2 % taurine	4·3	17·26	0·79
9	18	4. Control	6·3	27·01	1·82
		5. +0·5 % *S*-methylcysteine	6·7	29·21	1·98
10	17	6. Control	3·9	17·03	0·70
		7. +0·25 % cystine	5·7	32·95	1·98
		8. +0·25 % homocystine	4·5	23·07	1·08

In Exp. 8 equimolecular proportions of *S*-methylcysteine and taurine were administered and these compounds have clearly exerted no influence on liver fat deposition. Neither the percentages of fat in the liver, 16·48 and 17·26 respectively, nor the weights per 100 g. rat, 0·79 g. in both cases, are different from the control values, 16·36 % and 0·68 g. This inactivity of taurine was confirmed in a further experiment which will not be presented here in full; suffice it to say that the percentages of glyceride in the control and taurine groups were respectively 13·24 and 13·04 while the weights per 100 g. rat were 0·57 and 0·60 g.

Bearing in mind that it was not possible to demonstrate a convincing preventive action of methionine when the control group contained about 15 % fat in the liver, the next experiment with *S*-methylcysteine was designed to provide optimum conditions for the exertion of a preventive action. Accordingly, in Exp. 9 the 8 % egg albumin diet which invariably produces very fatty livers was used, and the control liver fat values were 27·01 % and 1·82 g. Here again, *S*-methylcysteine has exerted no effect on the liver fat for the values, 29·21 % and 1·98 g., do not differ significantly from the control figures.

One experiment was carried out with homocystine and the results indicated that this amino-acid behaved in a manner qualitatively similar to that of cystine. Whereas 0·25 % cystine produced a spectacular rise from the control values of 17·03 % and 0·70 g. to 32·95 % and 1·98 g., 0·25 % homocystine gave values of 23·07 % and 1·08 g. Further study of the action of homocystine was not made, since Singal & Eckstein [1939] and du Vigneaud *et al.* [1939] later found that homocystine markedly increases liver fat deposition.

Blood fat

Table 8 shows that the feeding of rats fasted for 24 hr. with a 40 % fat diet causes no changes in the amount of fat in the blood at any time up to 31 hr. after the meal, and, surprisingly, there is no indication of a blood fat curve or transient lipaemia. While changes in blood fat of many species of animals have been recorded in the literature, we have been unable to find observations on the rat with which the present results might be compared. The lack of any increase

in blood fat may be connected with the very slow rate of absorption of fat from the intestine with diets such as this, for we have repeatedly observed large amounts of unabsorbed fat in the alimentary tract many hours after feeding. It was this observation which led us to adopt the 16-hr. period after feeding for killing in the second experiment and even at this time the alimentary tracts of the majority of the animals still contain much unabsorbed fat. The results of the next experiment show very clearly the progressive increases of fat deposition in the liver with time.

Table 8. *Variations in blood fat of rats after a fatty meal and during the production of fatty livers*

Exp. 11

Group	No. of rats	No. of hours after feeding at which animals were killed	Total fatty acids in milli. equiv. per litre of whole blood
1	6	—	12·3
2	4	2·0	11·2
3	6	5·0	10·0
4	6	8·0	11·4
5	5	10·5	11·4
6	6	14·0	11·1
7	6	17·0	11·3
8	6	23·0	11·0
9	6	31·0	10·9

Exp. 12

Group	Period on fatty liver diet days	Added cystine %	"Fat" % fresh liver wt.	"Fat" in liver of 100 g. rat g.	Total fatty acids in milli. equiv. per litre of whole blood
Control	—	—	3·22	0·13	9·6
1*a*	1	—	4·69	0·16	11·1
1*b*	—	0·25	4·96	0·18	10·4
2*a*	3	—	8·74	0·30	9·3
2*b*	—	0·25	10·91	0·40	9·2
3*a*	7	—	14·22	0·56	9·2
3*b*	—	0·25	17·91	0·73	9·2
4*a*	14	—	23·91	1·06	9·0
4*b*	—	0·25	28·75	1·50	8·8
5*a*	21	—	27·08	1·36	8·9
5*b*	—	0·25	32·77	2·13	8·9

Thus the amount of fat begins to increase in the control groups within 24 hr. and progressively rises in 21 days from the zero figure of 0·13–1·36 g. on the 21st day. Similarly the effect of cystine is demonstrated at every stage, the amount of fat increasing progressively to 2·13 g. The effect of cystine appears to increase with time, for after 24 hr. it has caused a 12 % increase over the control and this steadily rises to 57 % after 21 days. In striking contrast to these liver fat changes, the blood fat figures show no change and it is noteworthy also that at every stage the blood fat of the cystine groups is identical with that of the controls. It may of course be that different results would have been obtained if the animals had been killed at a different hour after their last meal, but in view of the fact that much fat remained unabsorbed at this stage this seems unlikely.

Discussion

Table 1 shows clearly that the effect of cystine is demonstrated on 8% caseinogen diets containing as little as 0·05% added cystine. The daily intake of cystine from a diet containing 8% caseinogen would be about 3 mg., and the extra intake of 4 mg. added cystine profoundly increases the fat deposition. The results clearly show also that increasing the added cystine to five times this amount has no further effect on liver fat; this is in confirmation of the results previously reported [Beeston & Channon, 1936]. While the mode of action of cystine remains to be determined, the fact that its effect is unrelated to the intake is a factor which must be borne in mind.

The considerably increased fat deposition caused by cystine does not seem to be the result of a process differing from that which causes the deposition of fat in the livers of animals on the control high-fat diet but rather an acceleration of this process. This seems to be borne out by the fact that apparently no damage to the liver results, since the extra fat is removed readily by the same substances which normally prevent deposition, for 0·02% choline in the diet or 0·32 g. caseinogen per rat per day is sufficient to prevent the cystine action.

The results of Exp. 12 show that the acceleration of liver fat deposition caused by cystine cannot be associated with any changes in the amount of blood fat. This may mean that cystine exerts its effect in the liver itself, but at the present time no evidence exists which can serve as the basis of a theory to account for this action.

If it be assumed that methionine exerts a preventive action on liver fat deposition by supplying methyl groups for the synthesis of choline or of the active compound derived from choline, then three molecules of methionine would be required for the synthesis of one molecule of choline if complete conversion occurred; from this it follows that 3·3 mg. methionine should be equivalent in its action to 1 mg. choline. In Exp. 2 in which the amount of methionine used was such as to demonstrate the maximum action, 10 mg. methionine were equivalent to 2 mg. choline, i.e. a ratio by weight of 5 : 1. While this ratio derived from the results of Exp. 2 is the only certain finding on this question, support for it is given by the results of Exp. 1. In this experiment, 0·02% choline had an action equivalent to two-thirds of that of 0·20% methionine, and in the light of many previous experiments, it is reasonable to believe that 0·04% choline would have exerted an effect equal to this percentage of methionine. If this be correct, the ratio would again be 5 : 1. In view of the many difficulties of obtaining accurate assays in terms of choline, of substances having an action on liver fat, this ratio is to be regarded as being in quite reasonable agreement with the theoretical requirement, 3·3 : 1, and this finding provides, therefore, superficial evidence at least in support of the theory that methionine may act as a choline precursor.

On the other hand it must be emphasized that this ratio only obtains when the minimum amount of methionine required to exert maximum lipotropic action is considered. The fact that further increases above a certain minimum in the amount of methionine added to fatty-liver-producing diets exert no more action is one difficulty preventing the ready acceptance of this theory. In this respect methionine resembles cystine and differs from choline, the action of which is related to the amount administered. A second difficulty, probably associated with the first, lies in the fact that the action of methionine is limited in extent. In only one of our experiments has liver fat been reduced as low as 12% by methionine and when the control value is very high, methionine admini-

stration has not reduced it to a figure lower than 15%, and a liver containing 15% fat is still very fatty; in contrast, the use of a suitable amount of choline or lipotropically active protein will invariably reduce liver fat to well below 10%. These points can be explained in one of two ways; either methionine exerts its effect quite independently of choline or some factor other than the provision of methyl groups becomes limiting in the rate of synthesis of choline.

On the other hand our finding that *S*-methylcystine is without action lends support to the hypothesis of choline synthesis, for du Vigneaud *et al.* [1934] and Brand *et al.* [1935; 1937] showed that demethylation is probably not a stage in the metabolism of this compound. If this be true, *S*-methylcysteine could not supply methyl groups for choline synthesis. It must be remembered however that in the one experiment with homocystine reported here, this amino-acid increased liver fat deposition, having an action similar to but less intense than that of cystine. Further Singal & Eckstein [1939], in experiments on mice, find homocystine and cystine to be equally active in this respect, while du Vigneaud *et al.* [1939] also find that homocystine stimulates the deposition of liver fat. The primary product of the demethylation of methionine is homocysteine and the results of du Vigneaud *et al.* [1939] suggest that this compound is readily converted into homocystine. Hence in the demethylation of methionine to produce choline, there would be produced at the same time homocystine; the former compound would prevent fat deposition and the latter increase it. If the action of homocystine is like that of cystine, rising to its maximum at a very low level beyond which increasing amounts have no further effect, its action would be overcome as increasing amounts of choline were synthesized. This might account for the activity of methionine relative to that of choline being less than that expected from theoretical consideration.

Interesting observations on a problem of biological methylation have been made by Borsook & Dubnoff [1940]. These authors found that the conversion of glycocyamine into creatine by liver slices was increased some 50% in the presence of methionine but that neither any other amino-acid nor choline nor betaine had any effect. While methionine can provide methyl groups for the formation of creatine, choline is apparently unable to do so. The very different conditions of these experiments forbid their comparison with those of du Vigneaud *et al.* [1939] but the work provides definite evidence that methionine can act as a methylating agent in biological systems. Summarizing the findings presented in the present paper, it can be said that if methionine plays a part in the synthesis of choline, then some other dietary factor is also involved.

The results of these studies throw light on the question of whether the lipotropic action of proteins is solely dependent upon their cystine and methionine contents, as first suggested by Tucker & Eckstein [1937]. The fact that in Exp. 4 the extra weight of fat deposited in the liver as a result of the intake of the same amounts of added cystine progressively decreased as the content of dietary caseinogen was increased, strongly suggests, in view of the known lipotropic action of caseinogen, that the cystine present in proteins is intimately concerned in the production of fatty livers by dietary methods. In this connexion it is of interest to note also that in Exp. 4 group 2*b* which received 12% caseinogen with 0·25% added cystine gave the same results as group 1*a* receiving 8% caseinogen alone. It is possible therefore to equate these effects; the extra daily intake of 0·32 g. caseinogen by group 2*b*, as compared with group 1*b*, has thus neutralized the effect of cystine. Since the results of Exp. 3 showed that the cystine effect was prevented by 2 mg. choline, this finding shows that 1 g. caseinogen has an activity of approximately 6 mg. choline, a value in agreement

with that previously obtained [Beeston *et al.* 1936]. Thus whether the lipotropic action of caseinogen in terms of choline be derived from experiments concerned with fatty livers produced by diets of varying protein content or from those in which yet further fat is deposited in the liver as a result of cystine feeding, the same result holds. Furthermore, the results of Exps. 5 and 6 showed that the addition of cystine to basal albumin diets produced no further increase in the already large amount of fat deposited in the livers; similar observations when gliadin was the basal protein were reported by Tucker & Eckstein [1938]. Clearly, the cystine contents of these two proteins are so high that further supplementing exerts no effect. In our opinion, these findings are of importance in showing that the cystine content of dietary proteins is one of the factors normally concerned in dietary fatty liver production; we are gradually accumulating evidence, however, that cystine is not the only protein constituent acting in this way.

The limitations of the action of methionine have been discussed in detail already, and it is clear from what has been said that methionine cannot be solely responsible for the action of proteins in preventing liver fat deposition. That this amino-acid is of significant importance in this connexion is suggested by two facts; first, the proteins classified by Channon, Loach *et al.* [1938] in order of descending lipotropic activities, fall in the approximate order of descending methionine contents as Tucker & Eckstein [1938] pointed out; secondly, the latter authors showed that arachin, which contains very little methionine, has no lipotropic properties whatever.

On the other hand, results of the present experiments reveal the more complex nature of the problem. The lipotropic action of high fat diets containing caseinogen increases as the percentage of caseinogen is increased to 30% at which point the maximum effect of caseinogen is reached. The lipotropic action of the diets thus increases until caseinogen is providing to the diet 0·83% methionine. In contrast, while the results in Table 3 show that added methionine exerts an increasing effect up to 0·15–0·20%, those in Table 1 show that a further increase beyond 0·20% exerts no further effect. The limit of action is thus reached when the *total* methionine content of the diet is 0·42%. It does not seem possible therefore that methionine can be the sole active agent in the caseinogen molecule, a finding amply borne out by the results of Best & Ridout [1940]. These authors found that the administration of the amounts of cystine and methionine contained in 30% caseinogen exerted virtually no effect on fat deposition in contrast to the profound effect of 30% caseinogen itself. Since methionine is probably not the only lipotropic constituent of caseinogen, two possibilities arise; either that some other amino-acid also exerts a lipotropic action, or alternatively, added methionine is incapable of exerting its full action in the absence of some other protein constituent. In connexion with the former possibility previous work in our laboratory has shown that alanine, arginine, aspartic acid, histidine, hydroxyproline, leucine, lysine, proline and valine have no lipotropic action, although a slight action was observed with tyrosine [Beeston & Platt, 1939]. Thus of twelve amino-acids only methionine and to a far less extent tyrosine prevent fat deposition in the liver. We have no evidence bearing on the second possibility although we wish to mention that the production of aminoethanol, possibly from an amino-acid, might be the limiting factor. Among the number of compounds which have been reported to be active in the last few years, only one, betaine, seems likely to be of physiological importance, and it is interesting in this connexion that Carter & Melville [1940] found the betaines of serine, threonine and *allo*threonine to be inactive. It must not be

forgotten also that proteins may contain substances other than cystine which increase fat deposition.

It is clear that much further work is required before the lipotropic action of proteins can be explained. There are already two variable factors acting in opposite directions, namely cystine and methionine, and the logical deduction from the present evidence is that there are yet others. The isolation of the substances concerned is an essential preliminary and for this reason we are studying the action of fractions prepared from protein hydrolysates; results already obtained confirm the belief that lipotropic action of proteins is a far more complex story than the cystine-methionine hypothesis would suggest.

Summary

1. The greatly increased fat deposition in the liver caused by the addition of cystine to diets low in caseinogen and high in fat is seen when as little as 4 mg. of cystine are administered to the rat daily. No further increase results from increasing the cystine intake. The cystine effect can be demonstrated in progressively decreasing intensity at all levels of dietary caseinogen up to 25%. Dietary caseinogen exerts its normal lipotropic action on fat deposition caused by cystine; similarly 1·8 mg. choline per day will effectively prevent the cystine action. The increased fat deposition caused by cystine progressively increases with time up to 21 days. The cystine effect is not observed when the dietary caseinogen is replaced by albumin.

2. No changes were observed in the amount of fat in the blood of rats receiving diets high in fat and low in protein, either with or without added cystine.

3. The preventive action of methionine on liver fat deposition on an 8% caseinogen diet increases until the amount of added methionine is 0·20% of the diet; further additions of methionine to the diet do not have any increased effect. At its optimum, the intensity of action of methionine is one-fifth that of choline.

4. *S*-Methylcysteine and taurine are without effect on liver fat deposition; in contrast, homocystine has an action qualitatively similar to that of cystine.

5. The bearing of these findings on the respective modes of action of cystine and methionine in liver fat deposition and on the part played by these two amino-acids in accounting for the lipotropic action of proteins are discussed.

The authors wish to acknowledge their indebtedness to Dr V. du Vigneaud for a gift of *dl*-homocystine. Our thanks are also due to Mr G. T. Mills for carrying out cystine and methionine estimations.

The expenses of this research were defrayed in part by a grant from the Medical Research Council to whom we wish to express our thanks.

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106. FAT-DEFICIENCY DISEASE OF RATS. THE RELATIVE CURATIVE POTENCIES OF METHYL LINOLEATE AND METHYL ARACHIDONATE WITH A NOTE ON THE ACTION OF THE METHYL ESTERS OF FATTY ACIDS FROM COD LIVER OIL

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(*Received 9 March 1940*)

In a previous communication [Hume *et al.* 1938] experiments were described in which the relative potencies of methyl linoleate and linolenate in curing the fat-deficiency disease of rats were determined. The finding of Burr *et al.* [1932] was confirmed that the presence of either of these acids in a fat-free diet is able to promote the weight increase of rats but, in contrast with the results of Burr & Burr, it was found that linolenic acid had only about one-sixth of the potency of linoleic acid.

The highly unsaturated acids which have so far been shown to be characteristic of animal fat are the C_{20} acid (arachidonic) with 4 double bonds, and the C_{22} acid with 5 double bonds; it was shown by Nunn & Smedley-MacLean [1938] that arachidonic acid was absent from the livers of rats which had been fed for about 7 months on the fat-deficient diet, but was present in those which, after such a depletion period, had received for 5 weeks a daily dose of 14 mg. methyl linoleate.

Investigation of the activity of arachidonic acid was therefore of interest. Two apparently conflicting statements as to its biological activity appear in the literature. Burr *et al.* [1932] replaced 10% of a mixture of methyl linoleate and linolenate by methyl arachidonate, and found that the mixture containing the arachidonic acid was less effective than the original mixture. Turpeinen [1938], on the other hand, working in Evans's laboratory, claimed that methyl arachidonate was 3 times as potent as the linoleate in promoting weight increase, but he gave no detailed account of its effect on the skin condition. An investigation of the comparative effects of methyl linoleate and methyl arachidonate has therefore been carried out and is now described. The results appear to establish conclusively the superiority of methyl arachidonate in promoting weight increase but not in curing the skin lesions.

Preparation of methyl arachidonate.

The raw material used at first was pig's liver in which the highly unsaturated acid is mainly arachidonic [Hartley, 1909; Brown, 1928]. The freshly minced liver was added in 1 kg. portions to 2·5 l. of 4*N* HCl and the mixture boiled for 3 hr. in an atmosphere of N_2; the solid residue was then filtered off and

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heated with 10% alcoholic KOH for 1 hr. in an atmosphere of N_2, and part of the alcohol was then distilled off; the residual solution was acidified and extracted with benzene. The benzene solution of the fatty acids was dried for a short time with anhydrous Na_2SO_4, and Br was added in slight excess. The solid bromide which separated was extracted with ether and ethyl acetate; methyl arachidonate was obtained from it by debromination and esterification [Ault & Brown, 1934].

The accumulation in this way of a supply of methyl arachidonate sufficient for the feeding experiments would have been a laborious process, but it was subsequently possible to make more rapid progress through the kind help of Messrs Allen & Hanburys who furnished a supply of the crude fat extracted from ox suprarenal glands, which has been shown to be a rich source of arachidonic acid [Ault & Brown, 1934]. The crude fat was saponified with alcoholic KOH, and from this the acid was prepared as described above for pig's liver. The I.V. for the preparations of methyl arachidonate used for feeding were 312, 315, 320 and 290 (theory for $C_{21}H_{34}O_2=319$).

Biological technique

The biological technique was as described in the previous communication [Hume *et al.* 1938], with the following modifications.

Vitamins A and D were given in the form used in the second part of the previously reported investigation, vitamin D being given as irradiated ergosterol.

Vitamin E in the present experiments was given as *dl*-α-tocopherol, kindly supplied by Roche Products, Ltd. A 4% solution in paraffin oil, with the addition as stabiliser of 0·01% of ψ-cumoquinol (the gift of Prof. A. R. Todd), was given once a week, not later than the 9th week of experiment, in an amount corresponding to 0·8 mg. tocopherol, to all rats. The only available evidence that the dose was a sufficient one was the normal post-mortem appearance of the uterus, which was completely devoid of the brown coloration described by Martin & Moore [1939] as characteristic of even the virgin rat's uterus in vitamin E deficiency.

The rats received the fat-free diet from weaning, and dosage with the test material was begun not earlier than the 145th day and was continued for 5 weeks.

Both weight increase and cure of skin symptoms were used as criteria, and were assessed in the same way as before.

The rate of weight increase in rats receiving the fat-free diet, even when adequately supplemented with a more than minimum dose of essential unsaturated fatty acid, is not rapid, but it is not impossible that, after the long period of depletion, the normal rate of weight increase cannot be resumed [Clarke & Smith, 1938]. The possibility that insufficiency of choline may be acting as a limiting factor is being examined.

Animal experiments

For the comparison of methyl arachidonate with methyl linoleate 45 rats were used, of which 26 received supplements of arachidonate, 11 of linoleate and 8 served as untreated controls.

The experiment was carried out in two stages: in the first, daily doses of 42 and 14 mg. of the two materials were compared. The number of animals receiving 42 mg. of arachidonate had to be reduced to 2 through insufficiency of material, and the number receiving 14 mg. daily of arachidonate, therefore,

includes 5 which had received 42 mg. daily for the first 4 days of dosing. The results for weight increase and for cure of symptoms are set out in Tables 1A and 2A. The average values for weight increase with the larger dose are derived

Table 1. *Total weight increase (g.) in 35 days of young rats which had received a diet devoid of fat for about 140 days, and were then given various doses of methyl linoleate or methyl arachidonate or no dose*

			Methyl linoleate		Methyl arachidonate			
A								
Litter no.	Sex	Negative controls	Daily 42 mg.	Daily 14 mg.	Daily 42 mg.	Daily 14 mg.	Every 2nd day 14 mg.	Every 4th day 14 mg.
4591	♂	−15	31	.	.	37*	.	.
	♂	.	.	.	.	19	.	.
4599	♂	3	.	.	.	30*	.	.
4596	♂	11	.	.	.	.	.	.
	♀	6	32	18	.	25*	.	.
	♀	.	.	.	.	28	.	.
	♀	.	.	.	.	18	.	.
4600	♂	7	.	13	.	24*	.	.
	♂	.	.	.	.	16	.	.
	♀	9	.	11	.	17*	.	.
	♀	.	.	.	.	1	.	.
4604	♂	4	.	.	.	.	.	.
	♀	6	44	18	12	20	.	.
	♀	.	.	.	30	18	.	.
Average		6	35	15	21	21	.	.
B								
4619	♂	.	.	10	.	36	18	5
	♀	.	.	18	.	24	17	−6
4626	♂	.	.	.	.	33	7	4
	♀	.	.	20†	.	19	15	6
	♀	.	.	10	.	.	.	.
Average		.	.	14	.	28	14	4

* Had received 42 mg. daily for 4 days.
† Rat recovering from pneumonia.

from too few animals to give an indication. The average values for the smaller dose indicate some superiority of the arachidonate in promoting weight increase, but for cure of skin symptoms, the results for methyl linoleate appear if anything slightly superior.

In order to obtain if possible a more accurate quantitative result, a second experiment was made in which 2 litters of 8 rats each were divided equally into 4 groups of 4, of which 1 group received 14 mg. of linoleate daily, and the other 3 groups received 14 mg. (1 drop) of arachidonate every day, every other day and every 4th day, respectively (see Tables 1B and 2B). The average values for weight increase in this series, combined with the values obtained in the first series (Table 1A), indicate that the arachidonate had about twice the value of the linoleate. The average increase of 14 g. over the experimental period was the same for those receiving 14 mg. of linoleate daily, as for those receiving 14 mg. of arachidonate every other day; the values for the other doses fall sufficiently well into line with this result. It is to be noted that the response to 14 mg. daily of methyl arachidonate approached the maximal values obtained by the authors with the fat-free diet when supplemented with unsaturated esters.

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Table 2. *Healing of skin lesions of the ankles of rats which had received the fat-free diet for about 140 days, and were then dosed for 35 days with varying doses of methyl linoleate or methyl arachidonate or no dose. Same animals as in Table 1*

			Methyl linoleate		Methyl arachidonate			
A Litter no.	Sex	Negative controls	Daily 42 mg.	Daily 14 mg.	Daily 42 mg.	Daily 14 mg.	Every 2nd day 14 mg.	Every 4th day 14 mg.
4591	♂	θ	++	.	.	++*	.	.
	♂	.	.	.	.	+	.	.
4599	♂	θ	.	.	.	++*	.	.
4596	♂	θ	.	.	.	.	.	.
	♀	θ	+	+	.	+*	.	.
	♀	.	.	.	.	+	.	.
	♀	.	.	.	.	θ	.	.
4600	♂	θ	.	++	.	+*	.	.
	♂	.	.	.	.	+	.	.
	♀	θ	.	+	.	+*	.	.
	♀	.	.	.	.	+	.	.
4604	♂	θ	.	.	.	.	.	.
	♀	θ	++	+	++	θ	.	.
	♀	.	.	.	+	θ	.	.
B								
4619	♂	.	.	+	.	+	θ	θ
	♀	.	.	+	.	+	+	θ
4626	♂	.	.	.	.	+	θ	θ
	♀	.	.	+	.	+	θ	θ
	♀	.	.	+	.	.	.	.

Three degrees of healing are recognized: complete ++, partial +, none θ.
* Had received 42 mg. daily for 4 days.

For cure of skin symptoms in this second experiment also there was no superiority of the arachidonate; in 35 days, with a daily dose of 14 mg., healing was incomplete with both materials; with a dose of 14 mg. arachidonate every other day, there was no healing at all in 3 cases out of 4.

Prolonged action of methyl linoleate and methyl arachidonate

It was recorded in the previous communication [Hume *et al.* 1938, Table II] that the beneficial effect on weight increase and skin lesions of a daily dose of 80 mg. methyl linoleate could be observed long after the dose had been stopped. It seemed of interest to ascertain whether this was also the case with methyl arachidonate. The 16 rats used in the second experiment were therefore maintained on the unsupplemented diet for 12 days after dosage had ceased. The total weight increase for a group of 4 rats in the 12 days was greater for those which had received 14 mg. of linoleate or arachidonate every day than for those which had received this dose of arachidonate every 2nd or 4th day. Slight continued improvement of skin symptoms could also be detected in the rats which had received 14 mg. daily of linoleate or arachidonate, but with the others no improvement could be seen.

Action of cod liver oil esters in supplementing the fat-free diet

In the previous communication [Hume *et al.* 1938] it was reported that the methyl ester of the highly unsaturated acid, docosahexaenoic acid, isolated from cod liver oil by Farmer & van den Heuvel [1938], had little or no action in curing the skin lesions of the fat-deficiency disease, but that it did promote weight increase in rats whose weights had become stationary on the fat-free

diet. It seemed, therefore, to be of interest to ascertain what prophylactic action the whole fatty acid fraction of cod liver oil might have when given at the start of the experimental period on the fat-free diet.

Preparation of the methyl esters of cod liver oil acids. Cod liver oil was saponified and the methyl esters of the total fatty acids were prepared in the usual way. No division into saturated and unsaturated acids was made.

Biological experiment. Six rats, all females belonging to the same litter, received the fat-free diet as described above, but three of them received also in addition a daily supplement of methyl esters of cod liver oil acids. The amount was gradually increased from 1 drop (0·019 g.) at the beginning of the experiment to 4 drops by the 37th day, at which amount it remained until the 150th day when it was increased to 8 drops. On the 230th day whole cod liver oil was given instead of the methyl esters until the 260th day when the animals were killed. On the 230th day the weights of the 3 animals receiving the supplement of esters were 169, 174 and 174 g., and of the negative control animals 156, 161 and 166 g. The animals with supplements began to develop this slight advantage within the first few weeks of the experiment, and maintained it throughout.

All of the animals, without exception, showed dryness of the ankles, and all of them, except one receiving cod liver oil esters, showed ringing of the tail. The potency of the cod liver oil esters against the fat deficiency disease was thus very slight, and seemed to be less than that of the methyl ester of docosahexaenoic acid, but it should be remembered that the maximum dose of cod liver oil esters was about 150 mg. daily, while that of the pure docosahexaenoate was 60–100 mg. The docosahexaenoate constitutes not more than 5% of the total methyl esters of cod liver oil acids, so that the dose of docosahexaenoate given with the cod liver oil esters was very small.

Summary

1. Methyl arachidonate was prepared from pig's liver and from ox suprarenal glands.

2. The material was tested curatively on rats receiving the fat-free diet of Burr *et al.* in simultaneous comparisons with methyl linoleate.

3. In confirmation of the work of Turpeinen, methyl arachidonate was found to be more active than methyl linoleate in promoting weight increase; its activity in curing skin lesions was, however, no greater than that of methyl linoleate.

4. Methyl arachidonate, like methyl linoleate, unless given in very small doses, continued to exercise a beneficial effect for some time after dosage had been suspended.

5. Methyl esters of cod liver oil fatty acids were given prophylactically to young rats on the fat-free diet. The activity in promoting weight increase and in giving protection against skin lesions was very slight.

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107. FAT-DEFICIENCY DISEASE OF RATS. THE EFFECT OF DOSES OF METHYL ARACHIDONATE AND LINOLEATE ON FAT METABOLISM, WITH A NOTE ON THE ESTIMATION OF ARACHIDONIC ACID

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(*Received 9 March 1940*)

A HIGHLY unsaturated acid containing 20 carbon atoms and four double bonds was isolated by Hartley [1909] from the fatty acids of the pig's liver as its octabromide and named by him arachidonic acid. Subsequently the same acid was shown by Levene & Rolf [1922] to occur as a constituent of lecithin and kephalin, and since that time it has been shown to be widely distributed in animal tissues. In the ox, it was identified among the fatty acids of the liver, both in the neutral fat and in the phospholipin fraction; it occurs also in the acids from the fat depots and in the phospholipins of the heart, spleen and adrenals [Klenk & v. Schoenebeck, 1932], whilst Klenk & Dittmar [1936] and Wesson [1924] identified it among the brain fatty acids. Wesson [1925] estimated its proportion in the various tissues and endeavoured to determine the part played by this acid in metabolic processes.

Burr & Burr [1930] showed that the presence of highly unsaturated acids was essential for normal growth in rats and that these acids were not synthesized in the animal organism but had to be supplied in the diet. They found that either linoleic or linolenic acid could provide the missing factor, and that when either of these was included in the diet, normal growth was resumed. Wesson [1925] in his experiments had used corn starch as a constituent of the fat-deficient diet: this however on hydrolysis with acids yields appreciable quantities of linoleic acid [Taylor & Nelson, 1920] so that the essential fatty acid was actually being included in the diet of these fat-starved rats. It was also then not known how very slowly the body is depleted of the essential acids and that only very long-continued experiments will produce the symptoms of fat deficiency.

Burr *et al.* [1932] replaced 10% of a mixture of linoleate and linolenate by arachidonate and found that the relative increase of weight of rats receiving this mixture diminished when compared with rats receiving the full linoleate and linolenate supplements. It is however probable that the effect of a 10% substitution would give results within the biological variation of the experiment.

Turpeinen [1938] found methyl arachidonate to be three times as potent in producing weight increase as methyl linoleate; he did not specifically describe its effect on skin symptoms in the different rats but, speaking generally, said that the skin symptoms met with in the laboratory were mild. He measured however the number of ovulation cycles and found these to be markedly less than normal in the fat-starved rats but to be increased when arachidonate or

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linoleate was fed. He put forward the tentative hypothesis that the primary need of the body was for arachidonic acid.

Hume *et al.* [1938] showed that methyl linoleate was much more effective as a curative agent than methyl linolenate and Nunn & Smedley-MacLean [1938] established that arachidonic acid was not present in the liver fat of rats fed on a fat-free diet; when however the fat-starved rats received daily doses of methyl linoleate for a period of 5 weeks, arachidonic acid again appeared in the liver fat.

Burr *et al.* [1939] stated that they had found linoleic acid to be more useful in curing and preventing fat deficiency than either linolenic, arachidonic or the other higher unsaturated acids in fish oils.

Having in co-operation with E. M. Hume and H. H. Smith determined the effects of supplements of arachidonate on the growth and on the skin-condition of rats after a long period on a fat-free diet, we proceeded to investigate the fat of these rats. In our first experiment we examined the livers, kidneys and samples of muscle of four groups of these rats, determining the total content of lipoid matter, the percentage of phospholipin and the amounts of arachidonic acid measured by the weight of benzene-insoluble bromide formed on bromination in the phospholipin and neutral fat respectively.

Twenty-nine rats whose growth has been already described [Hume *et al.* 1940] were divided into 4 groups:

Group I. Eight rats which for 216 days after weaning had received only the fat-free diet.

Group II. Seven rats, of which after 163 days of the fat-free diet three received a supplement of 42 mg. and four, one of 14 mg. linoleate daily.

Group III. Six rats which, after receiving the fat-free diet for 163 days, then were given in addition, a supplement of 14 mg. methyl arachidonate for 39 days before being killed.

Group IV. Eight rats, six of which after the 163rd day of the fat-free diet received a daily supplement of 14 mg. arachidonate and two of which received daily 42 mg. arachidonate for 47 days before being killed.[1]

Method of preparation of phospholipin. Immediately after death, the livers, kidneys and samples of muscle from the lower limbs and body-wall were removed and the similar organs of each set of rats worked up together. The organs were sliced and dehydrated by immersion in acetone for 24 hr.; the dried residue was then twice extracted, each time for 1 hr. with boiling alcohol in an atmosphere of N_2 and finally with ether in a Soxhlet apparatus until no more fat could be extracted. The solvents were removed under diminished pressure and the residues extracted with ether. The ethereal solution was concentrated and after drying with anhydrous Na_2SO_4 was precipitated with acetone to remove the phospholipin fraction.

The degree of purification of the phospholipin varied with the amount available. When there was sufficient, it was precipitated four times by acetone from ethereal solution, the precipitate twice emulsified with a few drops of dilute NaCl solution and precipitated from this by acetone according to the method described by H. MacLean [1914]. The centrifuge was used to separate the precipitates and the ethereal solution was finally centrifuged to remove any deposit. The division into neutral fat and phospholipin is not an accurately quantitative separation but the results are comparative and should show any marked differences. Where the percentage of phospholipin was very small the neutral fat after the first separation of phospholipin was again dissolved in ether, and excess of acetone added to ensure complete separation. If the quantity of ether used as solvent had been small and the acetone in considerable excess, the amounts of phospholipin separated by reprecipitation were negligible. The degree of purity of the phospholipin was checked by phosphorus determination.

[1] The rats in groups I, II and IV, 4 hr. before being killed each received an injection of radio-active phosphate supplied by Prof. Hevesy.

Estimation of arachidonic acid. After saponification of neutral fat or phospholipin by boiling for 1 hr. with 10% alcoholic KOH, a current of nitrogen being passed during this operation, the free acids were extracted with ether and the ethereal solution dried overnight with anhydrous Na_2SO_4; the residue from the ethereal solution was dissolved in benzene and bromine carefully added to the ice-cooled solution.

After standing overnight at 0, the insoluble bromide which had separated was filtered off, washed with a few drops of benzene and then with ether until the washings gave only a trace of solid when a drop was evaporated on a watch-glass. The insoluble residue was then heated with 5 ml. benzene in a centrifuge tube in a water-bath at about 80°, the mixture centrifuged, the benzene solution decanted and the process repeated two or three times until the supernatant liquid contained only a trace of solid. Finally the residue after decanting the solution was washed with a little ether, centrifuged, the ether poured off and the centrifuge tube with the insoluble bromide dried to constant weight in a vacuum desiccator. Where the weight of bromide precipitate was more than about 12 mg. the percentage of Br was determined.

Although only about one-sixth of the theoretical amount of octabromide was precipitated when arachidonic acid, prepared by the debromination of the ether-insoluble bromide, was brominated in benzene solution of approximately 2% concentration, this proportion was found to vary but slightly and twice the weight of crude bromide 2 gave a very fair approximation to the amount of arachidonic acid present. Applying this to the mixed acids from the crude suprarenal fat and working at varying dilutions of from about 2% to 0·1% of arachidonic acid, we obtained reasonably constant results.

On washing with benzene as described in the method of estimation given above, about one-third of the precipitate was removed, and we felt it was justifiable to take the amount of arachidonic acid present as being approximately represented by 3 times the weight of benzene-insoluble bromide.

On further investigation we have found that the natural and debrominated acids show marked differences in the solubilities of their bromides and these results are discussed in the final section of this paper. We have therefore given the actual weights of benzene-insoluble bromides obtained under as far as possible comparable conditions. These weights probably represent about one-third the weights of arachidonic acid present. The method, whilst fairly satisfactory in the case of the natural acid, will give a low figure where the mixed fatty acids contain a small proportion of the debrominated acid which has been fed to the rats.

Results of exp. 1

The results of the analyses are shown in Table 1, Groups I–IV. The proportions of fat contained in liver, muscle and kidney tissues agreed closely in all four groups of rats, the percentages calculated on the weights of wet tissue lay in the kidney and liver between 3·1 and 3·5%: in muscle between 3·5 and 4·0%.

No significant variation in the proportions of phospholipin and neutral fat occurred: the values in the rats receiving only the fat-free diet were similar to those in the animals which had received the supplements of unsaturated acids. In most cases the lipoid matter from the livers contained approximately 50% phospholipin and 50% neutral fat, the greatest variation being between the two groups which had both received supplements of arachidonate but for varying periods. In the muscle the proportion of phospholipin in the lipoid matter varied from 6 to 13% but the differences could not be correlated with the presence or absence of the unsaturated acids.

The proportion of phospholipin in the kidney fat of the rats which had received the arachidonic acid supplement was much lower, but the amounts analysed were very small and this result would require further confirmation before it could be regarded as significant.

Amounts of arachidonic acid present in the organs of fat-starved rats. Bromination of the phospholipin acids derived from the liver of the negative controls

Table 1. *Showing the amounts of fat and phospholipin in organs of rats on various diets*

Group

I. 215 days on fat-free diet alone

II. 163 days on fat-free diet alone
+60 days with 1 drop linoleate daily

III. 163 days on fat-free diet
+39 days with 1 drop arachidonate daily

IV. 163 days on fat-free diet
+47 days with 1 drop arachidonate daily

V. 163 days on fat-free diet alone
+35 days with 1 drop arachidonate daily
+14 days on fat-free diet alone

Group

VI. 163 days on fat-free diet alone
+35 days with ½ drop arachidonate daily
+31 days on fat-free diet alone

VII. 163 days on fat-free diet alone
+35 days on ¼ drop arachidonate daily
+40 days on fat-free diet alone

VIII. 163 days on fat-free diet alone
+35 days with 1 drop linoleate daily
+12 days on fat-free diet alone

Group	No. of rats	Wt. wet tissue g.	Wt. dry tissue fat-free g.	Wt. neutral fat g.	Wt. phospho-lipin g.	% P in phospho-lipin	% P lipin in total lipin	% total lipin in wet tissue
Liver								
I (−ve)	8	61·8	—	1·00	1·00	3·55	50·0	3·24
II (1 L)	7	51·0	—	0·86	0·93	3·61	51·9	3·50
III (1 A)	6	46·0	—	0·69	0·87	3·12	55·8	3·40
IV (1 A)	8	61·0	—	1·00	0·92	3·39	44·6	3·40
V (1 A) (−ve)	4	35·0	—	0·70	0·41	3·23	36·9	3·17
VI (½ A) (−ve)	4	35·5	8·43	0·60	0·34	3·12	30·0	3·24
VII (¼ A) (−ve)	4	32·9	8·15	0·79	0·53	3·22	40·2	4·00
VIII (1 L) (−ve)	4	35·2	—	0·67	0·76	2·90	53·1	4·06
Wistar rats on normal diet:								
IX	4	25·8	5·17	1·29	—	—	—	5·00
X	4	24·2	5·06	0·87	—	—	—	3·60
Muscle								
I (−ve)	8	98·9	—	3·24	0·41	3·02	11·2	3·7
II (1 L)	7	111·2	—	3·92	0·27	3·07	6·2	3·9
III (1 A)	6	100·7	—	3·04	0·46	2·90	13·1	3·5
IV (1 A)	8	114·5	—	4·28	0·34	2·79	6·6	4·0
V (1 A) (−ve)	4	52·5	—	2·31	0·25	2·70	9·8	4·9
VI (½ A) (−ve)	4	52·7	12·1	3·42	0·46	2·83	11·8	7·4
VII (¼ A) (−ve)	4	58·6	11·8	2·50	0·29	3·07	9·0	4·3
VIII (1 L) (−ve)	4	57·0	—	2·29	0·34	2·90	12·9	4·6
Wistar rats on normal diet:								
IX	4	40·6	9·7	—	—	—	—	3·0
X	4	30·1	6·8	—	—	—	—	4·5
Kidney								
I (−ve)	8	17·5	—	0·34	0·20	2·77	37·0	3·08
II (1 L)	7	14·4	—	0·32	0·16	3·13	33·3	3·40
III (1 A)	6	—	—	—	—	—	—	—
IV (1 A)	8	17·2	—	0·44	0·10	3·28	18·5	3·14
V (1 A) (−ve)	4	7·8	—	0·35	0·04	1·74	10·2	5·62
VI (½ A) (−ve)	4	8·7	—	0·09	0·34	2·00	21·0	4·90
VII (¼ A) (−ve)	4	8·4	1·97	0·18	0·05	2·64	21·7	2·74
VIII (1 L) (−ve)	4	7·8	2·07	0·12	0·01	2·79	7·7	1·76
Wistar rats on normal diet:								
IX	4	4·2	0·68	0·26		—	—	6·20
X	4	4·3	—	0·20		—	—	4·70

gave only 4·4 mg. of a bromide insoluble in ether and benzene which decomposed above 240° without melting, and which may therefore be regarded as consisting chiefly of octa- or deca-bromide though the amount was too small for a bromine determination. The amount was, however, less than one-tenth of that isolated from the rats which had received the supplement of linoleate or arachidonate. The muscle phospholipin of the negative control rats also gave a small quantity

of insoluble bromide (6·2 mg.). No benzene-insoluble bromide was precipitated on brominating the fatty acids of the neutral fat.

The hexabromide (M.P. 202°) isolated from the livers of rats fed for 257 days on a fat-free diet and described in our previous communication [1938] was not identified, but the period of feeding with the fat-free diet in the present work was only 215 days and the longer period was probably necessary to ensure the complete exhaustion of the arachidonic acid.

After 215 days of the fat-free diet, small amounts of arachidonic acid still remained in the phospholipin of the liver and muscle tissues. In our previous work [1938] we found that after 257 days the liver was completely denuded of this acid.

In the present experiment the total increase in weight of 8 rats on the fat-free diet from the 163rd day to the 215th day on which they were killed, was only 28 g.: an average of less than 0·5 g. increase per rat per week. Since at the end of 31 weeks of the fat-free diet, the proportions of neutral fat and phospholipin were normal in liver, kidney and muscle tissue, there is no evidence that there is any failure in the processes by which these substances are formed in the fat-starved rat although the power to increase in weight has been lost. It must be remembered that during this period of depletion the rats have a constantly diminishing content of arachidonic acid, and until this is completely exhausted it cannot be assumed that this acid plays no part in the metabolic processes which are taking place in the organism. Our results support the view of Sinclair [1938] who studied the phospholipin metabolism of rats fed with elaidic acid and concluded that phospholipins containing only oleic and the saturated fatty acids serve in transporting fat and favour the combustion of fatty acids in the body.

In the rats fed with supplements of linoleic and arachidonic acids evidence of the occurrence of arachidonic acid was obtained in the neutral fat of the liver but considerably more occurred in the phospholipin fraction. In muscle fat

Table 2. *The weights of benzene-insoluble bromide derived from the arachidonic acid in phospholipin and neutral fat*

The amount of arachidonic acid is probably represented approximately by three times the weight of the benzene-insoluble bromide, precipitated in benzene solution and washed.

		In phospholipin			In neutral fat	
		Wt. of bromide			Wt. of bromide after washing with benzene mg.	% Br
Group	No. of rats	(*a*) As ppted in benzene solution mg.	(*b*) After washing with benzene mg.	% Br in (*b*)		
			Liver			
I (−ve)	8	—	4·4	—	0	—
II (Linoleate 60 days)	7	62·7	43·5	66·1	12·5	—
III (Arachidonate 39 days)	6	105·6	68·8	67·4	<1	—
IV (Arachidonate 47 days)	8	61·3	44·7	64·6	17·0	69·5
			Muscle			
I		—	6·2	—	<1	—
II		—	4·2	—	20	68·4
III		28·0	25·0	68·2	5	—
IV		—	8·0	—	10	—

arachidonic acid occurred both in neutral fat and phospholipin. The amounts of arachidonic acid are much greater in the phospholipin from liver than in that from muscle.

In our second experiment, we investigated three groups, each containing 4 rats; after 163 days on the fat-free diet, the rats in each group received per rat a supplement of methyl arachidonate consisting of 1 drop daily, 1 drop every other day and 1 drop on every 4th day in the three groups respectively for a period of 5 weeks, thus constituting doses of 1, 0·5 and 0·25 drop respectively for the rats in each group. The rats then again received only the fat-free diet until they were killed. A fourth group of 4 rats received a daily supplement of 1 drop linoleate during the 5-week period.

In order to determine the degree of fatness, and to avoid subjective impressions, we extracted and weighed the fat from the various tissues making in all about 80 % of the total weight. Neglecting the paws, head and tail we divided each group of rats into 6 fractions: (1) livers, (2) muscles from the hind limbs, (3) kidneys, (4) skin, (5) carcases, and (6) omentum with viscera. The carcase fraction included bones, muscles, thorax and the abdominal deposits of fat lying round the kidneys and on the back muscles.

On opening the abdomens of the rats which had received the sub-curative doses of arachidonate (0·5 and 0·25 drops) the animals appeared particularly well fattened, indeed they seemed to be loaded with fat and this appearance was confirmed by the weights of fat extracted. The figures are set out in Table 3, groups VI and VII.

The effects of small doses of the unsaturated acids on fat storage

In the liver. The addition of small doses of the essential unsaturated acids to the diet did not appear to have any very marked influence on the storage of liver fat. Whereas the percentages of fat obtained from the two similar groups of rats fed on a normal diet were 3·6 and 5·0, the percentage in the ten groups examined in our two experiments lay between 3·2 and 5·0. The only variation which might be regarded as significant was in the proportion of phospholipin to neutral fat in the three groups which had received respectively doses of 1, 0·5 and 0·25 drop arachidonate and had then returned to the fat-free diet. In these the percentage of phospholipin was between 30 and 40, being lowest in the livers of the "0·5 drop" rats.

In all the other groups examined the phospholipin percentages lay approximately between 45 and 55 %. The return to a fat-free diet after receiving for 5 weeks daily doses of arachidonate seemed to be associated with a diminution in the liver phospholipin content but not in that of the total lipin.

In muscle tissue. In three of the four groups now under investigation the proportion of total lipoid matter in the muscles was between 4·5 and 4·9 %, but in the rats which had received the 0·5 drop doses, the muscles contained 7·4 % of total lipin and showed a striking increase in both phospholipin and neutral fat contents. The proportion of muscle fat in all four groups seemed distinctly greater than in those which had continued to receive the doses of unsaturated acids until they were killed. In all groups the percentage of phospholipin varied from 9·0 to 12·9, agreeing with the values found in our first experiment.

In the skin. The proportion of total lipin is greatest in the rats which had received the 0·5 and 0·25 drop doses. Calculated on the fat-free dry weight the skin of the "0·5 drop" rats gave 62·2 %, the "0·25 drop" 51·9 % and the "1 drop" rats 43·0 % total lipin. It is again seen that the skins of the rats receiving the smaller doses are exceptionally well fattened as compared with

those of the rats which had received the 1 drop doses of arachidonate, and that as in the case of the muscles the proportion of skin fat is greatest in the rats which have received the 0·5 drop doses. This high percentage of fat in rats which after a short period of dosing had for nearly 6 weeks been living on the fat-free diet is striking.

In the kidney. The amount of fat extracted from the kidneys of each group of 4 rats was very small and was not separated into neutral fat and phospholipin nor was the percentage of phosphorus in the total lipin determined. The percentages of total lipin in the rats which had received the doses of 1 and 0·5 drop arachidonate followed by the fat-free diet were respectively 5·0 and 4·9 (cf. Table 1), agreeing with the values found in the groups of two normally fed Wistar rats (4·7 and 6·2 %); on the other hand the values in the groups of rats which had received the doses of 0·25 drop arachidonate and 1 drop linoleate were 2·7 and 1·7 %, i.e. less than in any other group investigated. No obvious correlation between the amounts of fat and the experimental conditions was visible and we felt that in order to investigate the kidney lipin satisfactorily, it would be necessary to use a larger number of animals in each group, so that more material would be available.

In the carcase. Quite abnormally large amounts of lipin were extracted from those rats which after receiving the 0·5 and 0·25 drop doses of arachidonate had been again transferred to the fat-free diet. Unfortunately in the working up of the fat from the corresponding group of rats fed with the 1 drop doses a small amount of fat was lost, not more than 5 % of the whole. Compared with two Wistar rats which had throughout received a complete diet, the rats which had received small doses of arachidonate for 5 weeks before being returned to the fat-free diet showed abnormal fattening in the inverse order of the amounts of arachidonic acid that they had received. Thus the carcases of the two groups of Wistar rats fed on a complete normal diet contained 35·7 and 38·0 parts total lipin for each 100 g. of fat-free dry tissue—compared with 40·8, 54·1 and 55·2 % for the rats which had respectively received the 1, 0·5 and 0·25 drop doses.

In the viscera. Since no fat had been contained in the diet of any of these rats, the omentum with the spleen, stomach, intestines and their contents were removed, weighed and the total lipin extracted and separated into neutral fat and phospholipin.

The superfatted condition of the rats which had only received for 5 weeks the subcurative doses of arachidonic acid and had then subsisted for nearly 6 weeks again on the fat-free diet, was a great surprise to us.

In Table 3 are set forth the total amounts of neutral fat and phospholipin extracted from the various groups. The wet weight of the tissues had not been recorded in all cases, and only in the group receiving the 0·25 drop doses, are the wet weights of all the tissues examined, available. It will be seen in this case that the latter represented 82 % of the total weights of the rats at the time of killing.

The crude phospholipin separated from carcase, skin and viscera was always small in amount and very impure since the phosphorus percentages lay between 1 and 2. The amounts of phospholipin from these organs shown in Table 3 have been recalculated and are given as the amounts which would contain 3·8 % P. The smaller adjustment necessary has also been made in the figures for liver, kidney and muscle.

During the 5 weeks period of dosing, the 4 rats fed with the 0·25 drop doses had made a total gain of only 9 g. in weight and another 8 g. during the following 38 days on the fat-free diet. But as shown in Table 3 their tissues contained

Table 3. *Showing total amounts of neutral fat and phospholipin in the 4 groups*

A = arachidonate L = linoleate

	Group V Dose for 5 weeks 1 drop A daily 14 days – ve			Group VI Dose for 5 weeks 0·5 drop A daily 38 days – ve		
	Wet wt. tissue g.	Neutral fat g.	Phospho-lipin g.	Wet wt. tissue g.	Neutral fat g.	Phospho-lipin g.
Liver	35·0	0·7	0·38	35·5	0·7	0·27
Sample of muscle	52·5	2·4	0·18	52·5	3·5	0·34
Carcase	—	32·9 +	0·61	—	45·6	2·00
Kidney	7·8	0·6	0·02	8·7	0·2	0·03
Skin	—	18·8	0·27	109·6	22·4	0·27
Viscera	79·4	8·1	0·11	83·9	8·9	0·22
		63·5 +	1·57		81·3	3·13
Total wt. fat + phospholipin		65·07			84·43	

	Group VII Dose for 5 weeks 0·25 drop A daily 40 days – ve			Group VIII Dose for 5 weeks 1 drop L daily 12 days – ve		
	Wet wt. tissue g.	Neutral fat g.	Phospho-lipin g.	Wet wt. tissue g.	Neutral fat g.	Phospho-lipin g.
Liver	32·9	0·9	0·44	35·2	0·9	0·50
Sample of muscle	58·6	2·6	0·23	57·0	2·4	0·30
Carcase	361·0	48·7	1·06	—	32·4	0·56
Kidney	8·4	0·4	0·04	7·8	0·1	0·01
Skin	109·8	19·0	0·14	—	19·0	0·08
Viscera	84·0	7·6	0·14	—	7·2	0·11
	654·7	79·2	2·05		62·0	1·56
Total wt. fat + phospholipin		81·25			63·56	

Table 4. *Showing total weight of 4 rats in each group*

Dosing started on 163rd day after weaning and ended on 198th day and rats then returned to fat-free diet.

Rats in Group V killed on 212th day—in Group VI on 236th day, in Group VII on 238th day and in Group VIII on 214th day.

	Group V "1 A" g.	Group VI "½ A" g.	Group VII "¼ A" g.	Group VIII "1 L" g.
On 163rd day	677	726	778	660
On 198th day	789	783	787	709
When killed	812	795	795	723
Gain in wt.:				
During dosing period	112	57	9	49
During subsequent period on fat-free diet (14 days)	23	(38 days) 12	(40 days) 8	(12 days) 14
Total gain	135	69	17	63

about 16 g. more fat than those of the rats which had received the 1 drop doses of arachidonic or linoleic acid for a similar period. Their total increase in weight therefore corresponds almost exactly to the excess of fat put on during the 5 weeks period of dosing and the subsequent 40 days on the fat-free diet. The effect of the smallest doses of arachidonate is therefore to store up fat in the fat deposits and there is no evidence that there has been any formation of new

tissue. It may be noted that in this case almost the whole of the increased weight of fat is stored in the fat deposits of the carcase.

The "0·5 drop" rats contained 3 g. less fat in their carcases than the "0·25 drop" rats, but muscle, skin and viscera contained more fat, so that there was in all about 18 g. more fat in the tissues examined than in those of the rats which had received the 1 drop doses of arachidonate or linoleate. The total weight increase in this group is however 69 g. of which 57 g. were put on during the period of dosing and 12 g. in the 38 days on the fat-free diet which supervened. About 50 g. of tissue which is not fat have been added with the increase in the dose of arachidonate and must presumably have represented an increase of new tissue provided by the formation of new cells.

The 4 rats receiving the 1 drop doses of arachidonic acid showed a weight increase of 112 g. during the dosing period, and of 23 g. in the following 14 days on the fat-free diet. These rats were well fattened but did not show the excessive fattening characteristic of the rats receiving the smaller doses.

These results indicate that the first effect of the arachidonate is to load up the connective tissue cells with lipoid matter especially in the perinephric fat of the carcase and that subsequently this excess of fat disappears as active growth occurs in the tissues.

The total weights of the rats in the three groups receiving the doses of arachidonate did not vary very widely (Table 4) and the total amounts of neutral fat and phospholipin extracted from the different groups may therefore be regarded as roughly comparable. The total phospholipin is markedly higher in the rats which have received the 0·25 and 0·5 drop arachidonate doses, and this is almost entirely made up of the excess in the carcase. While the excessive filling up of the fat depots is taking place the phospholipin increases proportionately more than the neutral fat, providing fresh evidence that the phospholipin is concerned in the transport of fat to the fat depots.

It is also interesting that in the lipoid matter from the livers of the rats fed entirely on the fat-free diet and in those which have received doses of the unsaturated acids continuously until they were killed the phospholipin forms from 42 to 52 % of the total lipoid matter. However, in the rats which after a long period of fat starvation received for 5 weeks doses of arachidonate and then returned to the fat-free diet, the phospholipin content of the total lipoid matter varied from 30 to 40 %.

A comparison of the two groups of rats which received 1 drop doses for 39 days and were then killed with those receiving doses for 35 days and then returning for 12 days to the fat-free diet is particularly interesting. Unfortunately only the figures for liver and muscle are available. These show a marked fall in liver phospholipin for the rats transferred to the fat-free diet and a marked increase in the percentage of neutral fat in the muscle tissue.

The proportion of arachidonic acid in the fat deposited

The proportion of arachidonic acid in the tissues of the rat as indicated by the weights of benzene-insoluble bromide formed on bromination varied greatly according to the amounts contained in their food. In rats which had been fed continuously on a normal diet containing adequate amounts of fat with the essential acids, the proportion of arachidonic acid in the lipoid matter extracted from the various tissues was high, especially in the liver. The amounts of fat derived from the small weights of kidneys available were so small that generally we did not attempt to analyse them further.

Table 5 shows the effects of the various diets on the amounts of benzene-insoluble bromides obtained per g. of total lipin. The figures given are certainly comparable and when multiplied by 2·9 probably give a fair indication of the weights of arachidonic acid in the lipin.

Table 5. *The influence of diet on the proportion of arachidonic acid found in the total lipoid substance (fat and phospholipin) from rat tissues*

The figures given represent the number of mg. of octabromide, precipitated on bromination of the fatty acids in benzene solution, the precipitate being subsequently extracted with benzene. The actual amounts of arachidonic acid are probably nearly equal to the figures given multiplied by 3.

	1		2		3
	Rats fed 163 days on fat-free diet, then received supplements of unsat. acids until they were killed		Rats fed 163 days on fat-free diet, then received supplements of unsat. acids for 35 days followed by fat-free diet		Rats fed for 215 days on fat-free diet and then killed
Liver	1 A (39 days)	44·2	1 A (14 days)	12·6	2·2
	1 A (47 „)	33·4	½ A (31 „)	4·4	
	1 L (60 „)	24·6	¼ A (40 „)	5·3	
			1 L (12 „)	8·5	
Muscle	1 A (39 days)	8·6	1 A (14 days)	3·9	1·7
	1 A (47 „)	3·9	½ A (31 „)	3·4	
	1 L (60 „)	5·8	¼ A (40 „)	3·9	
			1 L (12 „)	2·3	
Carcase			1 A (14 days)	2·8	
			½ A (31 „)	1·2	
			¼ A (40 „)	1·5	
			1 L (12 „)	0·9	
Skin			1 A (14 days)	1·0	
			½ A (31 „)	0·13	
			¼ A (40 „)	0·05	
			1 L (12 „)	0·05	

1 A = 1 drop arachidonic acid daily.

½ A = 1 drop arachidonate every other day.

¼ A = 1 drop arachidonate every fourth day.

1 L = 1 drop linoleate daily.

The number of days in brackets in col. 2 indicates the number of days the rats were returned to the fat-free diet before being killed after the period of dosing was ended.

The livers of two groups of rats kept for 163 days on a fat-free diet and then given a daily dose of 1 drop arachidonate for periods of 39 and 47 days respectively, gave 44·2 and 33·4 mg. octabromide per g. of total lipin. These contrasted with a group which after the 163 days on the fat-free diet had received for 35 days a daily dose of 1 drop arachidonate and were then again given the fat-free diet until they were killed 14 days later: here the octabromide precipitate had fallen to 12·6 mg. A similar result was obtained with linoleic acid; after 163 days of fat-free diet, 8 rats received daily 1 drop linoleic acid for 60 days before being killed: their liver fat gave 24·6 mg. octabromide per g. fat. The liver lipin of rats fed with similar doses for 35 days and then kept for 12 days on the completely fat-free diet yielded only 8·5 mg. octabromide per g. lipin. The livers of the rats which had received the 0·5 and 0·25 drop doses of arachidonate followed by the return to the fat-free diet for 31 and 40 days respectively gave 4·4 and 5·3 mg. arachidonic acid per g. total lipin, but in those kept for 215 days on the fat-free diet the comparable amount of octabromide had fallen to 2·2 mg. We have previously shown that with long-continued fat-free feeding, no arachidonic acid was detected.

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In muscle lipin somewhat the same relationship holds but the differences between the amounts of octabromide in the different groups are much less and the return to the fat-free diet after receiving the unsaturated acids seemed to exert much less influence than in the liver.

For the carcase and skin lipins unfortunately the figures for only one group are available, the data for these not having been determined in the first experiment.

The percentages of bromine in the benzene-insoluble bromide from the carcase fat of the rats which had received the 0·25 and 0·5 drop doses were abnormally low. Thus the figures for the benzene-insoluble bromide from the carcase phospholipin of the rats dosed with the 1, 0·5 and 0·25 drops arachidonate were respectively 1 A, 6·5 mg. (not analysed): $\frac{1}{2}$ A, 44·5 mg. (63·6 % Br): $\frac{1}{4}$ A, 34·5 mg. (61·7 % Br) whilst for the neutral fat the figures were 1 A, 94·2 mg. (67·4 % Br): $\frac{1}{2}$ A, 11·2 mg. (not analysed): $\frac{1}{4}$ A, 39·9 mg. (70·2 % Br). Theory for arachidonic octabromide requires 66·7 %. There was no obvious reason why the two phospholipin results gave such low figures. Possibly some of the dihydro-arachidonic hexabromide previously described by us [1938] was present.

In the skin fat from the same group of rats, only traces of benzene-insoluble bromide were precipitated although the dry scurfy condition of the ankles of the rats which had received the one drop doses of arachidonate had been completely cured.

A comparison of the four closely similar groups of 4 rats which received the doses of unsaturated acids for 35 days, indicated that the rats fed with daily doses of one drop linoleate contained in the total tissues examined (approximately 70 % of the weights of the rats) less than half as much arachidonic acid as did those fed with the daily dose of one drop arachidonate. It is unfortunate that similar figures were not available for the rats fed only on the fat-free diet but in the earlier experiments only the liver and muscle fat was analysed. A group of 3 rats which had been kept for many months on a fat-free diet and which should have provided the necessary comparison was not available owing to accidental circumstances.

The total amounts of benzene-insoluble bromide obtained from the groups of rats fed with the 0·25, 0·5 and 1 drop doses of arachidonate respectively were not widely different. They were only slightly higher in the rats which had received the whole drop doses. This fact might in part have been contributed to by the fact that the "1 drop" rats were killed rather less than 2 weeks after receiving their last dose of the acid whereas 5 to 6 weeks had elapsed after the dosing period before the "0·5" and "0·25 drop" rats were killed and during the longer period on the fat-free diet, more of the arachidonic acid would presumably have disappeared.

The interpretation of the results based on the estimation of arachidonic acid as the benzene-insoluble bromide is complicated by the fact that in our experience the solubilities of the octabromides of the naturally occurring arachidonic acid and of that obtained by debromination of the arachidonic-octabromide differ considerably when the precipitation is carried out in very dilute solution. Precipitation of the insoluble bromide of the natural acid is not very much affected even when conducted in very dilute benzene solutions but the bromide of the debrominated acid is considerably more soluble. On the other hand when precipitating in ethereal solution, the bromide of the recovered acid is more insoluble than that of the natural acid. As we did not discover this until late in the investigation, it was not possible to work out a more satisfactory method and we realize that whereas in the cases of the carcase and skin the

Table 6. *Showing the mg. of benzene-insoluble bromide isolated from the tissues of rats*

After 163 days on fat-free diet the rats had received doses of 1, 0·5 and 0·25 drop arachidonate and 1 drop linoleate respectively and had then been transferred to the fat-free diet for varying periods before being killed. The tissues extracted formed about 70% of the total weights of the rats.

Supplement fed to rats	1 drop linoleate		1 drop arachidonate		0·5 drop arachidonate		0·25 drop arachidonate	
	Neutral	Phospholipin	Neutral	Phospholipin	Neutral	Phospholipin	Neutral	Phospholipin
Liver	3	9·2	6	6	2	3·3	2	7
Sample of muscle	1	4·8	7	2	5	8	7	4
Carcase	31	5·5	94	7	11·2	44·5	39·9	34·5
Skin	0	1	16	2·8	0	3	0	1
Total in:								
Neutral fat	35		123		18·2		48·9	
Phospholipin	20·5		17·8		58·8		46·5	
Total	55·5		140·8		77·0		94·4	
Intake of arachidonic acid during feeding			1762		958		480	

The weights of benzene-insoluble bromide × 3·0 give an approximate indication of the amounts of arachidonic acid present.

percentage of arachidonic acid in the fat is low, considerable quantities of the debrominated acid might be stored and escape detection.

It is possible that on debromination the arachidonic acid is partially or wholly isomerized. As shown in the preceding communication the debrominated arachidonic acid can certainly be utilized in the body; feeding experiments in which the natural and recovered acids may be compared are already in progress.

It appears likely that the inclusion of arachidonic acid in a phospholipin is a necessary stage of the process of increasing the fat in the storage cells. In the most highly fattened rats (the 0·5 and 0·25 drop doses) a large proportion of the carcase arachidonic acid was contained in the phospholipin fraction. This may be due to some part played by the arachidonic acid in the transport of fat to the depot cells or possibly in the passage of the fatty acids through the cell membranes into bodies of the connective tissue cells. It is possible that in this process the incorporation of arachidonic acid into the kephalin molecule may play a part.

Since nearly as much arachidonic acid was detected in the tissues of the "0·25 drop" rats as in those of the "0·5 drop" rats, the increase in tissue weight other than that of fat, which was shown only by the "0·5 drop" rats probably did not depend wholly on the level of arachidonic acid in the tissues. In the "1 drop" group the amount of arachidonic acid contained in the phospholipin fraction was markedly less than in the "0·25" and "0·5 drop" groups.

In order that growth should take place as shown by an increase of weight of the animal much greater than that represented by any increase in the weight of fat, apparently in addition to the storage of arachidonic acid in the body phospholipin, a large excess of arachidonic acid is necessary and since it was not detected by estimation as the benzene-insoluble bromide, it is unlikely that it was present as the naturally occurring acid. In the "1 drop" rats, which gave no evidence of excessive fattening but showed marked increase in tissue weight we found no evidence of storage of a large proportion of the 1762 mg. arachidonic acid which they had received, and it was therefore probably not present in the

body as the natural arachidonic acid. Possibly arachidonic acid is oxidized in a chain of reactions associated with some vital process or has been converted into some other substance essential for cell proliferation.

It is perhaps of interest in this connexion to recall that Evans *et al.* [1934] found that in the absence of the highly unsaturated acids, even when vitamin E was supplied male rats were sterile and ovulation in the female was hindered.

Two distinct processes in which arachidonic acid plays an essential part appear to us to be indicated by our results:

(1) This acid plays some essential part in the process by which the connective tissue cells of the fat depots are first loaded up with fat. The effect is most apparent in the abdominal depots. It is possible that the inclusion of the arachidonic acid in the kephalin molecule might play a part in this process, although there is no evidence on this point.

(2) After the connective tissue cells have been filled up with fat, growth takes place. This is associated with a disappearance of the excessive fat deposits and the intake of a large excess of arachidonic acid which is converted into other products and is not stored as such in the body.

Cholesterol metabolism

In view of the fact that the highly unsaturated acids occur in the blood largely in combination with cholesterol, we determined the unsaponifiable matter in the neutral fat extracted from the organs of the four groups of rats which had received the doses of unsaturated acids for 35 days before being returned to the fat-free diet. These results are shown in Table 7.

Table 7. *Showing weights of unsaponifiable matter in the total lipin of rats fed on fat-free diet and receiving doses of unsaturated acids*

	Group VIII	Group V	Group VI	Group VII
Dose	1 drop linoleate mg.	1 drop arachidonate mg.	0·5 drop arachidonate mg.	0·25 drop arachidonate mg.
Liver	169	145	257	192
Muscle	67	59	75	111
Skin	524	574	634	765
Carcase	472	484	504	491
Kidney	14	39	38	47
Total	1246	1301	1508	1606
Total weights of phospholipin in same organs	1·45	1·46	2·91	1·91

The amounts of cholesterol are somewhat greater in the rats which have received the smaller doses of unsaturated acids.

The urine of the rats receiving the 0·25 and 0·5 drop doses was tested for acetone with negative results.

Note on the estimation of arachidonic acid

The methyl arachidonate used in the feeding experiments described in the previous communication was obtained by debromination of the octabromides prepared by brominating the crude fatty acids of suprarenal fat in solution in ether or benzene. So far as was known the arachidonic acid prepared in this way was identical with the naturally occurring acid. Since arachidonic acid contains 4 double bonds, it is capable of occurring in 16 geometrically isomeric forms. The possibilities of rearrangement both on bromination and debromination are therefore considerable.

Brown & Ault [1930] found differences in the melting points of the methyl esters of the octabromides derived respectively from ox and hog brains which they suggested might be due to the formation of isomeric bromides. Bosworth & Sissons [1934] showed that at least three isomeric octabromides were formed when the unsaturated acids separated from butter fat were brominated in ethereal solution and washed with ether. They isolated three bromides: (1) insoluble in petrol and soluble in ether M.P. 162°; (2) insoluble in ether but soluble in benzene M.P. 208°; (3) insoluble in all three solvents M.P. 257°. The same three bromides were also obtained by brominating in cold petrol solution, but with melting points 5 to 10° lower than those obtained by bromination in ethereal solution. All three bromides contained the theoretical amount of Br, on debromination gave an acid of mol. wt. 306·5 and I.V. 332·3 and on reduction arachidic acid, M.P. 74.

Experiments were made to determine the relative amounts of bromide precipitated when the debrominated arachidonic acid was brominated in 2% solution in various solvents (Table 8). The results show that when brominated in petrol solution as much as 85% of the theoretical amount of octabromide is precipitated of which more than 80% is ether-soluble. In preparing the octabromide obtained from a mixture of unsaturated acids, it should be advantageous to brominate in petrol solution as so high a percentage of octabromide is precipitated. In the absence of linoleic acid, which also gives a petrol-insoluble bromide, this should certainly prove the best method of preparation. Bromination in benzene gives little more than half the yield obtained by brominating in ethereal solution and is not therefore an economical method of recovering the acid. It does however yield an octabromide free from possible hexabromides.

Table 8. *Showing effect of solvent on amount of octabromide precipitated from 2% solutions of arachidonic acid obtained by debromination*

Weight of arachidonic acid g.	Solvent	Volume ml.	Weight insoluble bromide g.	% of theoretical amount of octabromide
0·3044	Light petroleum B.P. 50–60	15	0·7796	85·1
0·2866	Ether	15	0·2566	29·1
0·4408	Ether	20	0·3896	29·3
0·2958	CCl_4	15	0·2618	29·4
0·3186	Benzene	15	0·3186	16·3

The bromides precipitated in petrol and in ethereal solutions, were each heated with 70 ml. ether for 1 hr., the solutions being then cooled to laboratory temperature and decanted. This process was repeated nine times, the amounts of the precipitates dissolved being given below.

	Amounts dissolved from ppt. formed in petrol solution mg.	Amounts dissolved from ppt. formed in ethereal solution mg.
First extraction	530·6	60·3
Second extraction	61·4	48·0
Third to sixth	55·6	55·6
Seventh to tenth	23·2	24·8
Total amount extracted	670·8	188·7
Residue	91·6	204·6

The residues were then each extracted three times with 50 ml. benzene, when 71·6 mg. of residue from the precipitate from the petroleum solution and 60·4 mg. residue from the precipitate from the ethereal solution remained.

302 mg. bromide from the benzene solution were then extracted eight times with benzene, being heated each time with 15 ml. benzene for 1 hr.

The first three extractions removed 108 mg. substance soluble in benzene: the next five extracts contained 25 mg. leaving 168·8 mg. residue.

From these figures the proportions and ether- and benzene-soluble bromides may be calculated as percentages of the theoretical amounts of octabromide.

In ...	(1) Petrol %	(2) Ether %	(3) CCl_4 %	(4) Benzene %
Total ppt.	85·1	29·3	29·4	16·3
Ether-soluble fraction	70·2	14·3	—	7·3
Benzene-soluble fraction	6·2	5·3	—	9·1
Benzene-insoluble fraction	8·7	9·7	—	—

From these figures it seems probable that the precipitated bromides differ chiefly in the proportion of ether-soluble bromide which they contain. At least four isomeric bromides seem to be present:

(1) Petrol-insoluble and ether-soluble.
(2) Petrol- and ether-insoluble.
(3) Ether-insoluble: sparingly soluble in benzene.
(4) Very slightly soluble in benzene.

Ault & Brown [1934] found that when methyl arachidonate was brominated in ether and the precipitate washed with ether, the weight of bromide precipitate, though only about 26% of the theoretical amount, bore a constant ratio to the original weight of acid brominated. Further, when the C_{20} fractions of the methyl esters of the original unsaturated acids were brominated and the arachidonate content calculated from the I.V. the proportion of bromide insoluble in ether was similar to that obtained from the debrominated acid. They considered therefore that the most probable estimate of the amount of arachidonate present was obtained by multiplying the weight of bromide precipitated in ether by the factor 1·29.

In estimating the proportion of arachidonic acid present in the small amounts of fat derived from the organs of the rat, the fractionation of the methyl esters appeared to us impracticable as a stage in the process. It was obviously more convenient to brominate the mixed unsaturated acids or if the process were not affected by the presence of the saturated acids to brominate the mixed fatty acids at once. With regard to the solvent used for bromination, ether seemed to us undesirable in the present investigation because, although the ether-soluble bromides of oleic and linoleic acids would be removed, the benzene-soluble hexabromides would be weighed with the octabromide of arachidonic acid. It was unlikely that linolenic hexabromide would be present but the presence of the hexabromide of the C_{20} acid previously described by us [1938] was possible and in another series of experiments we had isolated a hexabromide of a dihydroxy-C_{20} acid also soluble in benzene. In order to eliminate as far as possible these benzene-soluble hexabromides, we decided to carry out the brominations in benzene solution.

As the solid bromide precipitated in ether or in benzene represented only a fraction of the theoretical amount of octabromide, experiments were made to

establish the constancy of the fractions precipitated and to determine a factor which, when multiplied by the weight of insoluble bromide precipitated, would give the weight of arachidonic acid present.

Two procedures were adopted: (1) the precipitates were left 48 hr. in the cold room, filtered on weighed filter papers, washed with 5–7 ml. ether and dried in a desiccator to constant weight; (2) the bromine was added to the solution of the acid in a weighed centrifuge tube which was left for 48 hr. in the cold room, then centrifuged and the residue again centrifuged three times with 5 ml. ether and dried to constant weight. In some of the experiments a considerable excess of palmitic and oleic acids was added to the solution to give conditions comparable with those occurring when the fatty acids of the rats' tissues are brominated.

Three samples of acid were used in these estimations:

(*a*) the acid obtained by debrominating octabromide prepared from suprarenal fat and containing 68·3 % Br;

(*b*) a mixture of oleic and arachidonic acids prepared from a sample of methyl esters of unsaturated acids which had been purified by molecular distillation and which from the I.V. contained 41·34 % arachidonic and 58·66 % oleic acids;

(*c*) the crude fatty acids obtained from suprarenal fat.

The two chief difficulties encountered were: (1) the amounts of precipitates formed from similar quantities of the debrominated acid and of that present in the natural fat showed marked differences especially in dilute benzene solution; (2) the solubility coefficients of the "insoluble" bromides were sufficient to cause serious discrepancies in very dilute solutions.

In order to purify the mixture of fatty acids without using either high temperature distillation of the methyl esters or a process involving the addition and removal of bromine, we enlisted the help of Mr Carr (British Drug Houses) and Dr Mead undertook to distil the methyl esters of the unsaturated acids separated from the acids of suprarenal fat by the Pb salt-alchol process I.V. 169·5; the fractions obtained were as follows:

	Temperature ° C.	I.V.	Weight g.
1	65	—	0·61
2	75	—	6·55
3	85–90	—	9·61
4	85–90	183·2	12·38
5	85–90	178·6	12·52
6	90	214·0	5·9
7	100–110	222·3	2·08
8	120	—	1·50
9	120–160	99·6	4·80

Fractions 3, 4 and 5 distilling at 85–90° were colourless liquids remaining completely liquid after long standing in the cold room. In fraction 6, some solid separated and fraction 7 solidified completely. Fraction 4 was saponified: from its I.V. it consisted of 41·34 % arachidonic mixed with oleic acid, and this mixture of acids was used for the bromination experiments.

A comparison of the factors arrived at when using the three different samples of acid is given below. It will be seen that with the debrominated acid the amount of bromide precipitated in 0·3 % solution is much less than with the acid purified by molecular distillation and that in the former case the factors are very large and irregular.

Factor, calculated from weight of insoluble bromide precipitated in ethereal solution from acids purified by molecular distillation, and washed three times with benzene.

Arachidonic acid %	A Debrominated acid	B Acids from methyl esters after molec. dist.
1·2	$F=2·15$	$F=1·90$
0·3	1·90	2·14
0·1	3·08	2·29

The solubilities of the bromides obtained by brominating the suprarenal fatty acids and the debrominated arachidonic acid in benzene solution were compared. Four extractions, each with 15 ml. benzene, dissolved 133 mg. from 302 mg. of the latter, i.e. 44 %, whereas in the case of the bromide from the

Table 9. *Estimation of Factor F where* $F=\frac{\text{Wt. arachidonic acid}}{\text{Wt. insoluble bromide}}$.

Column A contains results where weighed amounts of debrominated arachidonic acid were used: in B, a mixture of oleic and arachidonic acids purified by molecular distillation of the methyl esters was used and the amount of arachidonic acid calculated from the I.V. of the mixture: in C the crude fatty acids from suprarenal fat were used and the amount of arachidonic acid calculated as 1·2 times the weights of bromide precipitated in 1–2 % ethereal solutions.

% arachidonic acid	Insol. bromide pptd in ether			Insol. bromide pptd in benzene and washed with ether		
	A	B	C	A	B	C
1·9–2·0 %	$F=1·13$	—	—	$F=2·14$	—	—
	1·39			2·47		
	1·24			2·27		
	1·12			2·39		
				2·23		
				2·66		
				2·32		
				2·24		
				2·03		
1·0–1·2 %	1·32†	1·26	1·30	2·83	2·88	2·0
0·3 %	1·44†	1·44	—	8·35	3·40	—
	1·39			7·12		
				10·10		
				13·00		
				7·42		
				9·75		
				6·73*		
				5·52*		
				5·39*		2·1
0·2 %	—	—	—	—	—	1·8
0·1 %	1·76†	2·16	—	1·14?	2·00	—

* Oleic and palmitic acids were added to the solution.

† A mixture of 59 % oleic and 41 % arachidonic acid used to correspond with acids obtained by molecular distillation.

suprarenal acids, 72 mg. out of 282 mg. were dissolved, i.e. 26 %. The precipitates were heated in each extraction for 1 hr. with 15 ml. benzene, the solutions cooled to laboratory temperature and decanted. The bromide from the debrominated acid was therefore more soluble in benzene than that obtained from the natural acid. The percentages of bromine determined by Dr Weiler were:

Suprarenal acid, benzene-insol. fraction 67·3 %: benzene-sol. 64·9 %.
Debrominated acid, benzene-insol. fraction 66·4 %: benzene-sol. 67·0 %.
Theory for arachidonic octabromide = 67·7 %.

The bromide formed in benzene solution contains therefore at least two bromides, one removed readily by extraction with hot benzene and the other only slightly soluble in benzene. Whilst bromination in benzene solution gives a fair approximation of the amount of the naturally occurring arachidonic acid, it is not satisfactory in determining the amount of the acid obtained by debromination of the octabromide; the two acids appear to differ both in the relative solubilities of their ether-soluble and benzene-soluble bromides.

In the case of the natural acid, the ratio of the ether-insoluble to the benzene-insoluble bromide differs considerably from that obtained with the debrominated acid. And whereas, in the case of the natural acid, the amount of bromide precipitated in benzene solution is only slightly less on greatly diluting the solution, the amount of that precipitated in ether diminishes markedly on dilution. With the debrominated acid, the reverse is the case, here the amount of ether-insoluble bromide is only slightly affected on dilution whilst that of the benzene-insoluble fraction rapidly diminishes.

Thus the ratio of the weights of insoluble bromides precipitated in ether and benzene from the natural acid were in 1% solution 2·12 and in 0·3% solution 2·35; the corresponding figures for the debrominated acid were 2·2 and 5·2.

From the evidence now obtained it seems probable that during the process of adding and removing bromine, some geometrical isomerization of the acid has occurred. When a mixture of the two forms of the acid is present the estimation presents considerable difficulty: on the whole the discrepancies are probably least when the bromide is precipitated in ether solution and then washed with benzene. It must be emphasized that when thus comparing the amounts of arachidonic acid in small quantities of fat, the brominations should be carried out with approximately the same concentrations of arachidonic acid and the amounts of washing should also be similar. Under these conditions a fair estimate of the relative amounts of this acid may be gained. In our experience the weight of bromide precipitated in ether and three times washed with benzene is approximately equal to half the weight of arachidonic acid present.

Summary

1. The percentages of neutral fat and phospholipin calculated on the wet weight of tissue in the liver, muscle and kidney of rats kept for 215 days on a fat-free diet showed no significant variation when compared with similar figures for rats which after 163 days of fat-free diet had received curative doses of linoleic and arachidonate for 5–7 weeks before being killed.

2. In rats fed for 35 days with doses of arachidonate (1, 0·5 and 0·25 drop) and then returned to the fat-free diet, the liver lipoid substance contained less phospholipin than in those in which the 1 drop doses had been continued.

3. The muscles of rats which had received daily 1 drop doses of arachidonate and linoleate and had then been again fat-starved contained more fat than the muscles of rats which had received similar doses up to the time when they were killed.

4. The tissues, especially skin and carcase, of rats which had received 0·5 and 0·25 drop doses of arachidonate for 5 weeks and had then been again fat-starved were much more fattened than those of rats which had received 1 drop doses of arachidonate or linoleate under similar conditions. The carcases of the 0·5 drop and 0·25 drop rats were also richer in phospholipin.

5. The total weight increase of 17 g. shown by the 4 rats receiving the 0·25 drop doses of arachidonate corresponded almost exactly with the excess

fat found in their tissues. The 4 rats receiving the 0·5 drop doses increased by 69 g. of which only 19 were represented by excess fat. With the "1 drop" rats there was normal fat storage and much increase of weight.

6. Arachidonic acid was absent from the neutral fat of rats fat-starved for 215 days but occurred in very small quantity in the liver and muscle phospholipins. The C_{20} hexabromide (M.P. 202–4°) previously isolated from the liver lipin of rats kept for 257 days on the fat-free diet was not identified.

7. The arachidonic acid stored in the organs of the rats receiving different doses of the unsaturated acids was estimated and compared with the intake of arachidonic acid.

8. The conclusion was drawn that a minimum intake of arachidonic acid is necessary in order that the cells of the fat depots may first be loaded up with fat. Subsequently there is a process of true growth which is accompanied by the disappearance of comparatively large quantities of arachidonic acid.

9. The changes in cholesterol metabolism were also determined.

10. The method of estimating arachidonic acid by precipitation of the insoluble bromide is discussed.

We desire to acknowledge the help given us by Mrs Dolby who carried out estimations of arachidonic acid, by Mr Downer, who was responsible for most of the bromine determinations and by Dr M. G. Macfarlane, in whose laboratory the phosphorus estimations were carried out. We are also much indebted to Dr Mead (British Drug Houses Ltd.) for undertaking the molecular distillation of the fatty acid mixture.

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108. THE SYNTHESIS OF PHOSPHOLIPIN IN RATS FED ON THE FAT-DEFICIENT DIET

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(*Received 9 March 1940*)

The determination of the activity of the lipin-P in rats which have received injections of radioactive sodium phosphate some hours before being killed has been used by various observers to throw light on the synthesis of the phospholipins. It has been established by this means that constant synthesis and degradation of phospholipins is taking place and that this process is much more active in the liver and kidney than in the muscles [Hevesy, 1940]. Similar results were obtained when elaidic acid was used as an indicator [Sinclair & Smith, 1937].

The injection of radioactive phosphate into the fat-starved rats provided a method of comparing their power of synthesizing phospholipin with that of rats which had received, in addition to the fat-free diet, supplements of the essential unsaturated acids. Three groups of the rats, described by Hume *et al.* [1940], were placed at our disposal for this purpose. Group II comprised rats dosed for 38 days with methyl arachidonate following 163 days on the fat-free diet: Group III contained 8 rats kept without fat for 215 days: Group IV included 7 rats which had received supplements of linoleic acid for 60 days before their death. 4 hr. before they were killed each rat was injected with 0·5 ml. of a solution of radioactive phosphate of known activity which could subsequently be compared with a known standard.

The phospholipins extracted from the muscles, kidneys and livers of these three groups of animals were purified by the method of H. MacLean [1914]. The hydrolysate of the phospholipin was taken to dryness, the residue ashed with conc. H_2SO_4 and made up to a known volume.

One-fifth of this was retained for estimation of the phosphorus by Brigg's modification of Bell and Doisy's method. In order to ensure better precipitation when little phospholipin-P was present, 5·5 mg. P were added as sodium phosphate to the remaining four-fifths of the solution and the total phosphate precipitated as magnesium ammonium phosphate. The radioactivities of equal weights of the precipitates derived from phospholipin and added phosphate were determined and from these were calculated the activities of equal weights of the precipitates derived only from phospholipin-P. For convenience of measurement when comparing the precipitates of the liver phospholipin phosphates from Groups IV and II the former was diluted 5·3 times and the latter 12 times. There was not quite sufficient of the active phosphate to give the seventh rat of those receiving linoleate its full dose and a compensating factor of 1·057 has therefore to be introduced into the calculation for this group.

Table 1. *Showing relative activities of phosphate precipitates from phospholipins*

	Liver				Muscle				Kidney			
	mg. Phospholipin-P	mg. Total P	mg. ppt.	Relative* activity	mg. Phospholipin-P	mg. Total P	mg. ppt.	Relative activity	mg. Phospholipin-P	mg. Total P	mg. ppt.	Relative activity
Group II arachidonate	20·06	25·56	212·4	375	7·12	12·62	102·2	96	2·55	8·05	57·8	650
Group III fat-free	28·40	33·9	260·7	455	9·92	15·42	116·6	143	4·37	9·87	67·2	810
Group IV lineolate	26·90	32·4	249·6	916	4·92	10·42	79·4	74	4·08	9·58	77·3	966

* Relative activity = percentage of standard preparation.

$$\frac{\text{Liver}_3}{\text{Liver}_2}=\frac{455}{375}\times\frac{339}{284}\times\frac{2006}{2556}=1{\cdot}14$$

$$\frac{\text{Muscle}_3}{\text{Muscle}_2}=\frac{143}{96}\times\frac{15{\cdot}42}{9{\cdot}92}\times\frac{7{\cdot}12}{12{\cdot}62}=1{\cdot}34$$

$$\frac{\text{Kidney}_3}{\text{Kidney}}=\frac{810}{650}\times\frac{9{\cdot}87}{4{\cdot}37}\times\frac{255}{805}=0{\cdot}89$$

$$\frac{\text{Liver}_4}{\text{Liver}_2}=\frac{916}{375}\times\frac{32{\cdot}4}{26{\cdot}9}\times\frac{2006}{2556}\times\frac{5{\cdot}3}{12}\times1{\cdot}057=1{\cdot}08$$

$$\frac{\text{Muscle}_4}{\text{Muscle}_2}=\frac{74}{96}\times\frac{1042}{492}\times\frac{712}{1262}\times1{\cdot}057=0{\cdot}97$$

$$\frac{\text{Kidney}_4}{\text{Kidney}_2}=\frac{966}{650}\times\frac{958}{408}\times\frac{255}{805}\times1{\cdot}057=1{\cdot}16$$

These figures show that the amount of radioactive P taken up in the phospholipins of similar organs of these three groups of rats is surprisingly constant. Only in the case of the muscle tissue of the fat-free rats is there any significant variation. The turnover of P in the muscle phospholipin in the rats receiving only the fat-free diet is about one-third more active than in either of the groups which have received the supplements of unsaturated acids. This result is of particular interest since the evidence from the investigation of the R.Q. of rats with fat-deficiency disease suggested that more fat was being burnt than in the normal rat. Thus Wesson & Burr [1931] showed that in such rats in the first hours following carbohydrate feeding, the R.Q. is well above 1, indicating formation of fat from carbohydrate and that rats in the early stages of the disease show assimilatory and basal metabolic rates 25% greater than normal. This result was confirmed by Burr & Beber [1937] who found a much higher metabolic rate for the fat-starved rats; they state that for 12–16 hr. a day the R.Q. of fat-starved rats remained above 1, but when the rats stopped eating the R.Q. dropped rapidly, indicating the burning of much fat.

Since in the experiments described by Smedley-MacLean & Nunn [1940] the proportions of fatty acids in the liver, kidney and muscle tissues of the rats suffering from fat-deficiency showed no diminution, it could be inferred that the formation of fatty acid from carbohydrate and its conversion into phospholipin proceeded normally. The results of the radioactive injections show that in animals almost devoid of the higher unsaturated acids there is no diminution in the turnover of P in the liver and kidneys and presumably therefore phospholipin is being synthesized as actively in the rat suffering from long-standing fat deficiency as in those with the more normal diet containing the essential unsaturated acids. In the muscle where, according to our present belief, a great part of the lipin fatty acid is split off and burnt the turnover of P is one-third as great as in the muscles of the rats receiving the supplements of the essential acids. Since the R.Q. indicates that fat is being burnt and since the basal metabolism is enhanced it is especially interesting to find that more rapid phosphory-

lation and dephosphorylation are taking place in the muscles of the fat-starved rats. This result is in agreement with the view advocated by Sinclair and other workers that a large proportion of the body lecithin is concerned in the metabolism of fatty acids.

Relative rates of turnover in different organs

The ratio of the specific activities of the liver lipin P and muscle lipin P is a rough measure of the rate of lipin P turnover in these organs. A more correct measure would be the comparison of the ratios of the specific activities of the inorganic and lipin P present in the organs. The comparison of the specific activities of the lipin P of various organs does not take into account the different permeabilities of the cells of the various tissues. Thus muscle has been shown to be less permeable to phosphate ions than are the cells of the liver.

Leaving out of account this factor the ratio of the specific activities of the lipin P of muscle, kidney and liver was calculated as 1 : 12 : 21.

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amount of citric acid which was isolated as methyl citrate and accounted for about one-third of the total acidity of the original aqueous extract for the longer periods of incubation. The acids not extracted by ether were converted into barium salts and separated by precipitation with alcohol. An account of these is reserved for a further communication.

In order to obtain information as to the acids present in sound wood, healthy Scots pine sapwood chips were subjected to similar treatment. Volatile acids were found and formic acid was actually determined in the mixture. From the ether-soluble acids, however, no citric acid could be isolated as the trimethyl ester, and it seems reasonable to conclude that it is not present in sound wood. In order to obtain quantitative figures for the amount of citric acid present after decay, determinations of citric acid by the pentabromoacetone method [Lampitt & Rooke, 1936] were undertaken on the aqueous extracts. These values were in each case higher than the amount of citric acid obtained by isolation as methyl citrate, as would be expected, but the determination on the extract from sound wood gave a small positive citric acid value, whereas in the isolation experiment none could be detected.

Although healthy wood may possibly contain small amounts of citric acid which has hitherto escaped detection, the more probable explanation is that some substance is present in sound wood which is not citric acid but which yields pentabromoacetone under the conditions of the citric acid determination. Whatever the explanation of this divergence it is quite obvious even from the determinations of citric acid that the growth of the fungus on the wood does actually give rise to citric acid, which is therefore a true metabolic product. So far as we are aware the production of citric acid by species of wood-destroying fungi has not hitherto been recorded.

It is noteworthy that although the rate of decay as indicated by the loss in weight of the wood is steady throughout the 6 months' incubation period, yet the titratable acidity has reached a maximum in 2 months and the citric acid yield is constant after 4 months. This may represent the establishment of a balance between production and decomposition of acid. It is possible that further increase of acidity beyond this level would render the *p*H of the medium too low for the fungus. If this were the case much higher yields of citric acid might be attained by allowing the decay to proceed in the presence of some substance which could buffer or neutralize the acid formed. Further work is contemplated to investigate this aspect of the problem.

Experimental

In the preliminary experiments designed to afford a general survey of the acid production by various species of wood-destroying fungi on liquid media the *p*H of sample flasks containing 30 ml. of Kepler's malt solution was measured at various periods after inoculation. By testing separate flasks at each interval instead of withdrawing material from single large flasks, the volume of nutrient liquid available for the growth of the fungi was not reduced in the later stages of the experiment, but of course slight variation between the growths in different flasks was inevitable and the results do not lie on perfectly smooth curves. In earlier experiments the *p*H was determined colorimetrically with a Hellige comparator but in later experiments the glass electrode was used. The results are collected in Table 1.

The points to be noted in these preliminary experiments are:

(1) All the fungi tested show an initial lowering of *p*H, but whereas the brown rots maintain the *p*H at a low value, with the white rots the *p*H passes

through a minimum value and then rises again, in some cases to the original value or higher.

(2) The depression in *p*H produced by the brown rots is considerable, the greatest being shown by *Coniophora cerebella* where a *p*H of 2·0 is attained after 10 weeks.

Table 1. *p*H *changes during growth on Kepler's malt solution*

Brown rot fungi

Period of incubation weeks	*p*H by comparator			*p*H by glass electrode		
	Merulius lacrymans	**M. lacrymans*	*Daedalea quercina*	*Coniophora cerebella*	*Lenzites trabea*	*Fomes pinicola*
0	6·8	6·4	5·8	7·4	5·6	5·6
1	6·6	6·1	4·65	7·0	4·7	4·05
1½	—	—	—	6·15	4·1	3·05
2	5·35	5·15	3·3	2·6	4·0	2·85
3	3·0	3·6	—	2·25	—	—
3½	—	—	2·8	—	3·5	2·7
4	2·9	2·3	—	2·25	—	—
5	—	—	2·8	—	3·1	2·6
6	2·0	2·2	—	2·2	—	—
7	—	—	—	—	3·05	2·55
8	—	—	2·4	2·35	—	—
9	—	—	—	—	3·1	2·65
10	—	—	—	2·0	—	—
11	2·6	3·0	—	—	3·15	2·55
12	—	—	2·8	2·1	—	—

White rot fungi

Period of incubation weeks	*p*H by comparator				*p*H by glass electrode			
	Polystictus versicolor	**P. versicolor*	*Stereum hirsutum*	*Fomes annosus*	*P. versicolor*	*Trametes rubescens*	*Ganoderma applanatum*	*Fomes ignarius*
0	6·8	6·4	5·8	5·8	7·4	7·4	5·6	5·6
1	5·45	5·3	4·6	5·3	6·8	6·9	5·55	5·5
1½	—	—	—	—	6·25	6·5	4·65	5·15
2	4·2	4·2	3·5	3·9	4·4	6·15	4·25	(5·4)
3	4·2	4·2	—	—	4·35	4·95	—	—
3½	—	—	4·0	3·8	—	—	4·35	4·05
4	4·2	4·0	—	—	5·45	5·25	—	—
5	—	—	4·0	3·8	—	—	4·3	4·25
6	5·8	6·0	—	—	6·55	5·95	—	—
7	—	—	—	—	—	—	(4·05)	4·6
8	—	—	4·2	4·6	7·15	5·7	—	—
9	—	—	—	—	—	—	5·25	4·25
10	—	—	—	—	6·65	5·55	—	—
11	6·7	7·2	—	—	—	—	7·25	4·5
12	—	—	5·0	5·6	7·3	5·65	—	—

Columns marked with an asterisk are for 5% malt solution, the rest are for 2% solution.

In view of its behaviour not only on liquid media, but also on wood, where twice as much acid was produced as was given by other species tested, the fungus *C. cerebella* was selected for a closer study and for the purpose of identifying the acid metabolic products.

Organism. A culture of *C. cerebella* Pers. originally isolated by Prof. R. Falck at Hann-Münden was used. This strain, which has been called the "*Idaweiche* form", has been extensively employed as a test fungus in toxicity tests of wood preservatives (British Standards Specification No. 838 of 1939).

Method of culture

The method used for cultivating the fungus was exactly similar to that already described for the examination of *Lentinus lepideus* [Birkinshaw & Findlay, 1940]. Small blocks of Scots pine sapwood sterilized by autoclaving for 30 min. were introduced into Kolle flasks in which the fungus was growing on malt agar. They were supported on low glass rests so that they came into contact only with mycelium thereby minimizing any diffusion of materials from the agar into the blocks. The flasks containing the samples were kept in an incubator at 22° and batches of samples were removed after 2, 4 and 6 months' incubation.

Method of examination of infected wood

The partly decayed wood was extracted and the water-soluble products were investigated after periods of incubation of 2, 4 and 6 months' in order to ascertain the changes in nature and amount of products as incubation progressed. The treatment of batch I was rather of an exploratory nature; the technique was modified for succeeding batches in the light of experience gained from batch I.

Treatment of batch I (2 months' incubation)

100 blocks of the infected wood (total moist weight 998 g.) were cut into chips and treated with 7 l. of cold distilled water. The container was evacuated until all the chips sank below the surface when air was readmitted. After 20 hr. the aqueous solution was removed and a second extraction was performed with 5 l. of water. After 24 hr. the extract was removed and the wood was pulped in a manual mincer. The pulp was pressed in a tincture press and extracted with a further 5 l. of water, the pulp being again pressed after extraction. Titration of 5 ml. samples of the extracts showed that the third extract had only about 1/7 of the acidity of the first extract. Further extraction was not considered profitable.

The total volume of the combined extracts, pressings and wash water was now 18,020 ml., the acidity by titration being equivalent to 182·4 ml. of *N*.

After removing a reference sample of 1/20 of the volume the remaining liquor was concentrated *in vacuo*. The distillate was collected and titrated for volatile acids. The total titratable acidity of the combined distillates was 118·8 ml. of *N*/10.

When cooled to 0° the dark brown concentrate deposited a dark-coloured sediment (2·2 g.; ppt. I) which was separated by filtration. The liquor was then neutralized to litmus by the addition of 85 ml. of 2*N* NH_4OH. A solution containing 30 g. of $CaCl_2,6H_2O$ was then added and the mixture left for 12 hr. in the cold. A slight precipitate was obtained; this was separated by filtration (0·39 g.; ppt. II).

The filtrate and washings (600 ml.) were treated with 2500 ml. of absolute alcohol to make an 80 % alcohol solution. The bulky precipitate was separated at the centrifuge, washed and dried (26·67 g.; ppt. III).

Concentration of the alcoholic liquor and reprecipitation gave only small amounts of sticky precipitates which were not further investigated.

Examination of the fractions obtained

Volatile acids. The combined distillates obtained on evaporation had been neutralized and required 118·8 ml. of *N*/10 NaOH. The solution was evaporated almost to dryness and the acids were liberated by means of phosphoric acid and redistilled *in vacuo*, in order to eliminate CO_2 which might have been present

in the first distillation. The titration of the distillate was now 89·8 ml. of *N*/10 NaOH.

The sodium salts were evaporated to dryness and a portion (0·1 g.) was converted into phenylphenacyl esters by heating with *p*-phenylphenacyl bromide (0·3 g.) in 6 ml. of 60 % alcohol under reflux for 90 min. The crystals obtained on cooling had M.P. 91–95°, raised by recrystallization to 107–108° and by vacuum sublimation to 109–110°. It showed no depression in M.P. when mixed with authentic *p*-phenylphenacyl acetate of M.P. 110–111°. (Found: C, 75·63; H, 5·80 %. Calc. for $C_{16}H_{14}O_3$, C, 75·58; H, 5·55 %.) The substance is therefore *p*-phenylphenacyl acetate and indicates the presence of acetic acid in the volatile acids. A further portion of the sodium salts of the volatile acids was found to give a heavy precipitate of metallic silver when treated with silver nitrate solution, indicating the probable presence of considerable amounts of formic acid. A definite identification of formic acid was obtained from the second batch.

Precipitate I. The product was found to contain material extractable by ether, the extract amounting to about 10 % of the total weight. This might be resinous material from the wood.

The main portion appeared to be a salt. It was acid to litmus, gave a strong potassium flame and had a sulphated ash value of 54·3 %. It was thought that it might be potassium hydrogen tartrate. However, by qualitative tests (Fenton's reaction, Feigl's fluorescein reaction [Feigl *et al.* 1936]), tartaric acid could not be detected. Further, a determination of tartaric acid by oxidation with periodic acid gave an apparent potassium hydrogen tartrate content of only 15·3 %. As this test is not specific even the low figure obtained may have been due to other substances. There is thus no definite evidence of the presence of tartaric acid in the precipitate and if present at all the amount must be relatively small. As this ppt. I was found to contain considerable amounts of iron—no doubt derived from the mincer or press—it was not further examined. In working up further batches care was taken to avoid these sources of contamination.

Precipitate II. Identification of oxalic acid. A portion of this precipitate (0·34 g.) was acidified with 2·5 ml. of 2*N* HCl and extracted with ether in a continuous extractor. The total extract (0·15 g.) was sublimed *in vacuo* and gave a crystalline sublimate of M.P. 189°. Freshly sublimed oxalic acid had M.P. 190° and there was no depression on mixing. Hence oxalic acid is definitely present in this precipitate but the total amount formed is relatively small since the total weight of ppt. II, which is presumably crude calcium oxalate, is only 0·39 g.

Precipitate III. (Wt. 26·67 g.) This, the largest fraction, was subjected to a more detailed examination. Various quantitative and qualitative tests were performed on the aqueous solution in order to obtain some idea of the nature of the product.

(1) *Sulphated ash content.* 32·15 %. Assuming that the only cation present is Ca this gives a Ca content of 9·46 %. Actually the ash was found to contain iron.

(2) *Estimations of sugar as glucose.* The apparent glucose content of the precipitate by the Shaffer-Hartmann method was 14·15 %. Another portion of the precipitate was hydrolysed by heating 0·2039 g. in 10 ml. of water with 20 ml. of *N* H_2SO_4 for 1 hr. at 100°. The apparent glucose content after hydrolysis (corrected for dilution) was 33·63 %. Thus the substance evidently contains polysaccharides or polyuronic acids. A qualitative test for polysaccharides was made by dissolving a little of the precipitate in dilute HCl and treating with 4 vol. of alcohol. A considerable amount of flocculent precipitate indicated the presence of polysaccharides.

(3) *Estimation of aldehyde groups* (Willstätter and Schudel). The precipitate (0·2492 g.) was dissolved in 100 ml. of water and treated with 40 ml. of $N/10$ iodine and 50 ml. of $N/10$ NaOH. After 15 min. 10 ml. of $2N$ H_2SO_4 were added and the solution was titrated with $N/10$ thiosulphate. The iodine deficiency (8·73 ml.) corresponded with CHO content of 0·0127 g. or a glucose content of 31·5 %.

(4) *Test with 2:4-dinitrophenylhydrazine.* An aqueous solution of the precipitate gave a precipitation with dinitrophenylhydrazine hydrochloride in $2N$ HCl after 4 hr. which gradually increased in amount over several days. No crystalline product could be obtained from this.

(5) *Test for hexuronic acids.* The precipitate was dissolved in water and an equal volume of conc. HCl was added together with a little naphthoresorcinol. On warming, a greenish colour appeared at 70°, steel grey at 80°. Amyl alcohol extracted a deep blue colour with green fluorescence. In ether a purple colour was obtained. The test indicates the presence of a uronic acid grouping.

(6) *Attempt to crystallize the Ca salts.* A portion of the precipitate was dissolved in water, filtered and the filtrate was allowed to evaporate slowly in a desiccator. The substance dried down eventually to a horny mass showing no trace of crystals.

(7) *Esterification of the acids.* The precipitate (5 g.) was dissolved in water and filtered from a small amount of insoluble matter. The dark brown solution was treated with a slight excess of oxalic acid and after removal of the calcium oxalate by filtration the solution was evaporated to dryness *in vacuo* (3·94 g.). The solid was treated with methyl alcohol, in which a part dissolved and was methylated with a solution of diazomethane in ether. It was then filtered from the insoluble portion (1·52 g.) and the solution was evaporated. The sticky residue weighed 2·40 g. Of this residue 0·05 g. was subjected to sublimation in high vacuum. A crystalline sublimate M.P. 72–75° was obtained. The remainder was extracted three times with about 50 ml. of ether under reflux. The extract, 0·60 g., was refluxed with light petroleum (B.P. 50–60°). This gave crystals on cooling of M.P. 77·5–78° unchanged after recrystallization. Further extractions and evaporation of the mother liquors gave further crops of crystals (0·25 g. in all) of slightly lower M.P. There was no depression in M.P. when the crystals were mixed with authentic methyl citrate of the same M.P. (Found: C, 46·72, 46·40; H, 6·14, 5·96; OCH_3, 39·4, 39·2 %. Calc. for $C_5H_{14}O_7$ (with $3OCH_3$), C, 46·15; H, 6·02; OCH_3, 39·8 %.)

For further confirmation the amide was prepared. The crystals (0·1 g.), M.P. 76–77°, were treated with 0·5 ml. of aqueous ammonia saturated at 0°. The ester dissolved, and on keeping crystals were obtained which, when recrystallized from water, had M.P. 206–207°. Pure citramide had M.P. 207–208°. There was no depression on mixing. (Found: C, 38·58, 38·50; H, 5·73, 5·88; N, 21·3, 20·9 %. Calc. for $C_6H_{11}O_4N_3$, C, 38·09; H, 5·86; N, 22·21 %.)

The methyl ester fraction soluble in methyl alcohol but not extracted by ether was treated according to Goebel [1935] for the preparation of the hepta-acetyl methyl ester of aldobionic acid. The ester (0·74 g.) was heated at 50° for 12 hr. under reflux with 5 ml. of acetic anhydride containing 0·5 g. of anhydrous $ZnCl_2$. The acetic anhydride was removed *in vacuo* and the residue was taken up in chloroform, washed 5 times with ice water, separated, dried with calcium chloride and filtered. It was then evaporated *in vacuo* and the residue was taken up in alcohol and chilled. No crystals could be obtained.

Treatment of batch II. (4 months' incubation)

As already stated the procedure employed in the examination of batch II was modified in the light of experience gained with batch I. The modified procedure was designed (1) to avoid contamination with iron, (2) to separate the polysaccharides before precipitation of the salts of the acids, (3) to obtain the barium instead of the calcium salts, since the former might be more readily separated by a process of fractional precipitation.

The second batch of decayed wood had a total moist weight of 853·5 g. It was cut into chips and extracted with cold water, the extractions being repeated until the titration value of the extract had fallen from 0·97 ml. of *N*/10 NaOH per 5 ml. (1st extraction) to 0·09 ml. (4th extraction). Mincing and pressing were avoided in order to prevent metallic contamination. The total volume of the extract was 18,660 ml. and the total acid titration value 167·8 ml. of *N*. One-twentieth of the mixed extracts was retained for reference and was preserved by the addition of a little toluene; the remainder was evaporated *in vacuo*, the distillate being retained for examination of the volatile acids.

The aqueous extract was evaporated to 300 ml. and the precipitate which formed was filtered off (1·14 g.). The solution was then treated with absolute alcohol sufficient to make 90 % by volume in order to remove the polysaccharides. The precipitate was separated by centrifuging (8·50 g.). The alcoholic solution was evaporated *in vacuo* to about 150 ml. During evaporation a small amount of sticky material separated. The aqueous solution of 150 ml. remaining after evaporation of all the alcohol and some of the water was then extracted in a continuous extractor with ether for 72 hr. After this period the amount of material still being extracted by ether was negligible. The extract, after removal of ether, weighed 12·43 g. This was dissolved in methyl alcohol. There was a small amount of insoluble matter (0·05 g.).

The aqueous solution which had been extracted with ether was treated with a filtered solution of 8·1 g. of $Ba(OH)_2,8H_2O$ in hot water, the amount required to neutralize the solution having been ascertained by titration of a small sample. The baryta was added slowly to the solution, which was mechanically stirred during the addition. The precipitate obtained was separated by centrifuging, washed and dried (0·88 g.).

The solution (370 ml.) was treated with an equal volume of absolute alcohol and the precipitate was again removed by centrifuging (7·21 g.). The alcohol was then made up to 80 % and the precipitate was separated (3·68 g.).

Batch II. Examination of the fractions obtained

Volatile acids. The total titration value of the distillate was 53·47 ml. *N*/10 NaOH. The neutralized solution was evaporated to dryness and the acids were redistilled after addition of 1 g. of phosphoric acid to the salts. The titration value of the distillate was now 38·27 ml. *N*/10. The neutralized solution was again evaporated and made up to 50 ml. A portion (5 ml.) showed a formic acid content of 9·73 mg.; thus the titratable acidity due to formic acid would correspond with 55·3 % of the total acidity.

The remaining solution (45 ml.) of the salts of volatile acid was distilled with aqueous phosphoric acid *in vacuo*. $PbCO_3$ (1 g.) was added to the distillate which was then warmed and filtered. The filtrate was evaporated to 2·5 ml. and chilled. Crystals were obtained (0·13 g.) in the form of long needles characteristic of lead formate. By sulphated ash the Pb content of the crystals was

69·9, 69·7 %. Calc. for $Pb(HCOO)_2$, 69·7 %. Thus the presence of formic acid in the distillate is definitely established.

All the residues and washings were collected, acidified with phosphoric acid and distilled. The distillate was titrated, and the neutralized liquid after concentration was treated with the calculated amount of *p*-phenylphenacyl bromide. Sufficient alcohol was added to maintain the bromide in solution and the mixture was refluxed for 1·5 hr. The products were fractionated from alcohol-water. Crystals were obtained having M.P. 108–109° which showed no depression in M.P. when mixed with authentic phenylphenacyl acetate. The presence of acetic acid in the volatile acids is thus again confirmed.

Treatment of acids extracted by ether. The total acids extracted by ether (12·43 g.) were dissolved in 65 ml. of methyl alcohol, the small amount of insoluble matter was separated by filtration and the solution was treated with diazomethane in ether until the evolution of nitrogen ceased. The product was evaporated to dryness and extracted with ether. The ether-soluble portion (9·79 g.), after removal of solvent, consisted of a mass of crystals in a syrupy matrix. This was spread on a porous plate. The syrup was slowly absorbed, leaving 4·21 g. of crude crystals. After two recrystallizations from light petroleum (B.P. 40–50°) the crystalline material showed the correct M.P. 77·5–78° for methyl citrate.

The substance absorbed by the porous plate was recovered by extraction and dried. It had acid equivalent on saponification of 121·8. Attempts to prepare from it a crystalline hydrazide, phenylhydrazide or amide failed.

Batch III. (6 months' incubation)

The treatment of batch III (moist weight 1128 g.) was substantially the same as for batch II and need not therefore be described in detail. The titration values, etc. of the total and volatile acids are recorded below:

Aqueous extract

Total volume of aqueous extract ...	19,550 ml.
Titration value	240·5 ml. *N* NaOH

Volatile acids

Titration value of distillate	50·23 ml. *N*/10
After redistillation	28·96 ml. *N*/10
% of acidity due to formic acid ...	26·8 %

Two separations of the polysaccharide fraction were undertaken, as in work on the previous batch this fraction had not been completely eliminated, before proceeding with the precipitation of the barium salts.

The evaporated aqueous solution (300 ml.) was treated with 9 vol. of alcohol and the polysaccharide precipitate separated (12·80 g.). After removal of alcohol the aqueous solution was exhaustively extracted with ether. The total weight of the ether extract was 16·4 g. The extracted solution was then again treated with 9 vol. of alcohol and the precipitated polysaccharides removed (2·75 g.). Further addition of alcohol up to 95 % caused no further precipitation. The alcohol was removed and the solution (now less than 100 ml.) was neutralized with baryta (9·11 g. of $Ba(OH)_2, 8H_2O$) and the Ba salts were fractionally precipitated with alcohol.

Isolation of citric acid as the methyl ester from the acids extracted by ether. The method was identical with that already described for batch II except that one-

quarter of the total acids extracted by ether was used instead of the whole amount. The amount isolated showed no increase over batch II.

Examination of sound wood

The treatment of the sound wood followed the method described for batches II and III of the decayed wood.

Chips of Scots pine sapwood (743 g.) which had been steam distilled for several hours were extracted three times with cold water as follows: 3·5 l. for 60 hr., 3·2 l. for 48 hr., 3 l. for 48 hr. The titration fell from 0·225 ml. of *N*/10 NaOH per 5 ml. for the 1st extraction to 0·053 ml. for the 3rd extraction. The total titration value of the mixed extracts was 25·9 ml. of *N* NaOH. The extract was distilled *in vacuo*, and gave an acid distillate of titration value 37·0 ml. of *N*/10. On redistillation of the distillate the titration was 24·74 ml. of *N*/10. The formic acid content was 69·0 mg. equivalent to 60·7 % of the total acidity of the volatile acid fraction. The evaporated aqueous solution containing the non-volatile acids was made up to 90 % alcohol and gave a polysaccharide fraction of 3·58 g. The solution was evaporated and deposited a little brownish oil. This was separated by filtration and the clear liquid was exhaustively extracted with ether. The whole of the extract was esterified with diazomethane and the solvent was evaporated. The product was extracted with ether, and gave an ether-soluble portion of 2·78 g. and an insoluble residue of 0·03 g. There was no crystallization in the syrup remaining after removal of the ether. A small portion of the syrup sublimed *in vacuo* gave no crystals but only an oil which distilled at 100°. In a further attempt to obtain crystals of methyl citrate the ether-soluble portion was extracted with light petroleum (B.P. 40–50°). The extract (1·40 g.) was still an uncrystallizable oil. Hence it may be concluded that citric acid is not present in sound wood. By a second precipitation with alcohol of the acid fraction not extracted by ether a further small amount (0·83 g.) of polysaccharides was obtained. The acids remaining after removal of the alcohol had a titration value of only 5·5 ml. of *N*/10 and gave 0·12 g. of barium salts. Thus practically the whole of the material obtained as barium salts from batches II and III of decayed wood may be regarded as metabolic products of the fungus.

Determination of citric acid

Citric acid was determined by the pentabromoacetone method as described by Lampitt & Rooke [1936] in samples of unevaporated aqueous extract of each of the three batches of decayed wood and also in a similar extract from sound wood. These figures are not of great accuracy since the amounts found in 100 ml. (the volume used for the determination) are near the lower limit of the method but they are interesting for comparison with the amounts of citric acid actually isolated as methyl citrate. The values obtained are given in Table 2, the amounts of citric acid being expressed in each case as the weight of anhydrous acid obtainable from 100 blocks of wood (estimated dry weight 793 g.). The dry weight of decayed wood after extraction is given for comparison in order to show the loss in weight owing to decay. Total and volatile acidities are also shown.

It is of interest to note that the control estimation of citric acid in sound wood gives a definite although small positive value, yet no citric acid could be isolated as methyl citrate. The reason for this anomaly is obscure; possible explanations have already been discussed.

Table 2. *Data obtained on wood extracts*

Wood sample	Dry wt. of wood after extraction g.	% loss in wt.	Total acid (ml. *N*) extracted by H_2O	Volatile acid (ml. *N*)	% of volatile acidity due to formic acid	Anhydrous citric acid: Determined by pentabromo-acetone method: Wt. g.	Anhydrous citric acid: Determined by pentabromo-acetone method: % of total acidity	Anhydrous citric acid: Isolated as Me_3 citrate: Wt. g.	Anhydrous citric acid: Isolated as Me_3 citrate: % of total acidity
Control	783	1·31	30·2	3·04*	60·7	0·44	22·8	0	0
Batch I (2 months)	669	15·64	182·4	9·46	40·2†	1·19	10·2	1·18	10·1
Batch II (4 months)	546	31·15	167·8	4·03	55·3	4·95	46·1	3·69	34·4
Batch III (6 months)	445	43·88	185·0	2·35	26·8	4·98	42·1	3·68	31·1

All yields are based on 100 blocks of wood of estimated dry weight 793 g. before inoculation.

* This value is almost certainly low owing to the material having been subjected to steam distillation prior to extraction.

† Known to be a low value.

Summary

From a comparison of various wood-destroying fungi grown on aqueous malt extract and on wood *Coniophora cerebella* was selected as the best acid producer. The acidic products formed by growth of *C. cerebella* on Scots pine sapwood were therefore investigated after incubation periods of 2, 4 and 6 months. The acids identified were formic, acetic, traces of oxalic acid and relatively large amounts of citric acid. The last-named acid was isolated as the methyl ester. It was obtained in amounts equivalent to about one-third of the total titratable acidity after 4 and 6 months' growth. Other acids of higher molecular weight were also present. Qualitative tests indicated the presence of hexuronic acids in this fraction.

Examination of sound wood revealed the presence of volatile acids including formic acid in amounts comparable with those isolated from decayed wood. The volatile acids are not therefore to be regarded as metabolic products of the fungus. Citric acid, on the other hand, is considered to be a true metabolic product of the growth of *C. cerebella* on wood.

Part of this research was carried out during the tenure of an Agricultural Research Scholarship of the Ministry of Agriculture by one of us (R. A. W.). We accordingly acknowledge our indebtedness to the Ministry and also to the Department of Scientific and Industrial Research since a portion of the work was undertaken as part of the programmes of its Chemistry and Forest Products Research Boards. We further wish to express our appreciation of the interest shown in this work by Prof. H. Raistrick.

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110. THE INFLUENCE OF THE LYOTROPIC SERIES OF ANIONS ON CATION PERMEABILITY

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(*Received 15 April 1940*)

THE literature contains the results of a great deal of work on the influence of the lyotropic series of ions on physiological phenomena. The idea in these studies was that cells, including their membranes, constituted colloidal systems, and since the most characteristic feature of colloids is the influence of hydration on their physical state, it was argued that if certain physiological phenomena showed influences by ions which normally affected the degree of hydration of colloidal systems, then the systems determining the physiological phenomena were in themselves colloidal. Thus Höber [1909] has studied the influence of anions on the ciliary movement in the epithelium of the frog's stomach, and has found that if the anions are arranged in the order of their activities a lyotropic series is obtained. Similarly, e.g. Port [1910] has studied the effect of anions on saponin haemolysis, Raber [1920] changes in the conductivity of *Laminaria* to alternating currents, Gellhorn [1922] changes in the motility of spermatozoa and Loeb & Cattell [1915] the poisoning of *Fundulus* embryos by K and the facilitation of recovery. It is on the basis of work of this nature that Höber [1922] has developed the so-called "Colloid-chemical Viewpoint" in regard to physiological phenomena; according to this viewpoint, the membrane of the cell is a colloidal system and its permeability is determined by its degree of hydration. Dehydrating substances, such as Na_2SO_4, were supposed to exert a thickening or packing effect on the micelles constituting the membrane, thereby decreasing permeability; hydrating substances, such as NaCNS, were supposed on the other hand to loosen the membrane and so increase permeability.

A careful investigation of the claim that SO_4 decreases permeability and that CNS increases it reveals that this is not a general rule; thus if attention is confined only to the work quoted by Höber [1922] it is found that Raber [1920] reports the reverse relationship and Loeb & Cattell [1915] show that whereas dehydrating substances such as SO_4 and tartrate apparently inhibit the penetration of K into *Fundulus* embryos, the same substances apparently accelerate the escape of this ion from the embryos. Again the evidence put forward by Port [1910] on the effect of ions on saponin haemolysis has not been confirmed by the more exact measurements of Ponder [1934], and Höber [1908] in his study of hypotonic haemolysis ignored the shrinking of the erythrocyte caused by the presence of a divalent anion, such as SO_4, through ionic exchanges.

In view of these considerations it appears that the experimental basis for the colloid-chemical viewpoint is not strong, and it is therefore of interest to study the influence of the lyotropic series of anions on a comparatively simple system. Such a system is provided by the erythrocytes of the cat which show a well-defined and accurately measurable permeability to the cations Na and K [Davson, 1940].

The cat erythrocyte, unlike those of most other species, contains mainly Na as cation, so that if it is suspended in isotonic KCl solution there will be a gradient of K in the direction: suspension medium → cells, and a gradient of Na in the opposite direction. Hence if cat cells are suspended in isotonic solutions of KCNS, KI, KBr, KCl etc., and the rates at which K enters and Na leaves the cells are measured, influences of the anions on cation permeability may be determined. Before passing to the experimental results, it may be pointed out that a series of anions may exert influences on cation permeability merely in virtue of the different diffusion potentials created, provided that the anions themselves are permeable. Thus, provided that the chances of Na-K exchanges across the membrane are negligibly small in comparison with the chances of the migration of a pair of oppositely charged ions, the rate at which a K salt of a univalent acid will enter the cell will be given by Nernst's equation:

$$D = \frac{2UV}{U+V} RT,$$

where U and V refer to the mobilities of the cation and anion through the membrane respectively. If V varies with the different anions, it is clear that the observed rate of penetration of K when cells are suspended in solutions of the K salts of different acids will vary in accordance with the mobility of the anion of the salt. Hence an influence of a series of anions on cation permeability is not necessarily to be attributed to an influence on the structure of the membrane, i.e. by hydrating or dehydrating it, and it may be that this purely electrical effect was a factor in the many studies of the influence of the lyotropic series on physiological activity, since the activity of tissues and cells is bound up in some way, not clearly understood, with the migration of ions across membranes.

The assumption that Na-K exchanges will be infrequent in comparison with the migration of pairs of oppositely charged ions is justified in the case of the cat erythrocyte, since the permeability of the membrane to Na and K is very small in comparison with its permeability to anions; also earlier results indicate that the penetration of K is virtually independent of the speed of penetration of Na and *vice versa*.

Experimental

Blood was taken from the cat under ether anaesthesia. Since storage of the blood overnight has been found to affect Na permeability, leaving K permeability unchanged, the blood was used on the day of withdrawal for experiments on Na permeability; in general experiments on K permeability were carried out a day later. The experimental technique for studying cation permeability was essentially the same as that described earlier [Davson, 1934; 1937]; in experiments involving K permeability the cells after suspension in a solution of a K salt were washed once with isotonic NaCl solution to remove interstitial K. This procedure was necessary and was shown not to produce any significant errors. Since 1·4 ml. of whole blood were added to 10 ml. of the solution of K salt, the cell suspension was in general adequately buffered, except in the case of the K salts of slowly penetrating acids, e.g. SO_4, where, owing to the exchange of anions from the interior of the cell with OH ions from the outside, the reaction shifted from pH 7·4 to pH 7·0; such a shift is sufficient to produce a serious change in Na permeability only, and this point has been discussed in the section on results. The solutions of the K salts of the fatty acids were prepared by titration of 0·165 M KOH with the purest specimens of the acids obtainable. All experiments were carried out in a bath at 25°.

Results

Potassium permeability. If the quantity of K penetrating the erythrocytes in 1 hr., when suspended in an isotonic solution of the particular K salt, is used as an index to the rate of penetration, a typical experiment gives the following series of permeabilities of the cell membrane to K, the value for KCl being put equal to 100:

CNS	I	NO_3	Br	Cl	Acetate	Oxalate	Sulphate	Citrate	Tartrate
178	143	133	112	100	85	84	56	45	29

i.e. the series is that known as the lyotropic series. However, it would appear from the work of Ege [1922] and of Maizels [1934] that the anions penetrate the erythrocyte in the same order as a lyotropic series, so that the effects observed here may be due to the electrical diffusion effects discussed earlier.

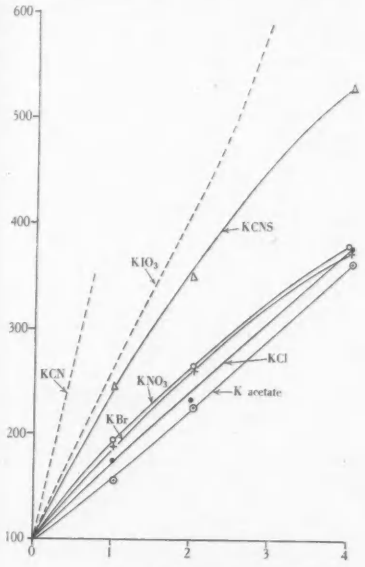

Fig. 1.

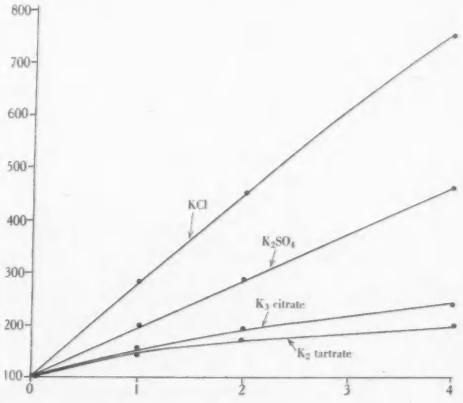

Fig. 2.

Fig. 1. Increase in the K content of cat erythrocytes suspended in isotonic solutions of the K salts of various acids. Ordinates: percentage of the original content. Abscissae: time in hours.

Fig. 2. Increase in the K content of erythrocytes suspended in solutions of the K salts of various acids. Ordinates: percentage of the original content. Abscissae: time in hours.

In Fig. 1 curves for the penetration of K from the K salts of a number of univalent acids are given; the curves in unbroken lines refer to experiments carried out simultaneously on the same sample of blood, and are therefore strictly comparable among themselves; those in broken lines refer to experiments on different samples of blood, and in plotting the results on the same scale as the others the curve for the penetration of KCl in each instance has been used as the standard of reference. The curves for Cl, NO_3, Br and acetate lie very

close together, whilst CNS, IO_3 and CN show very decided accelerations of K permeability in comparison with Cl. In Fig. 2 curves for multivalent anions are shown and it is evident that these anions retard K penetration in comparison with Cl. In these experiments the solutions were 0·165 *M* with respect to K and tonicity was maintained equivalent to that of a 0·165 *M* K salt of a univalent anion by addition of an appropriate amount of the Na salt of the anion investigated.

In an attempt to determine whether the effects of the anions observed here were due to alterations in the membrane or to the diffusion effects discussed earlier, the proportions of isotonic KCl and K_2 tartrate solutions in the suspension medium were varied, and the rates at which K entered the cells determined; similar experiments were carried out with Cl-SO_4, Cl-IO_3 and Cl-CNS mixtures. If the effect of the anion, other than Cl, is one on the membrane, a linear relationship between the concentration of this anion and permeability would not necessarily be expected, whereas if the effect were due to diffusion potentials alone, a linear variation would be expected. The curves (Fig. 3) for

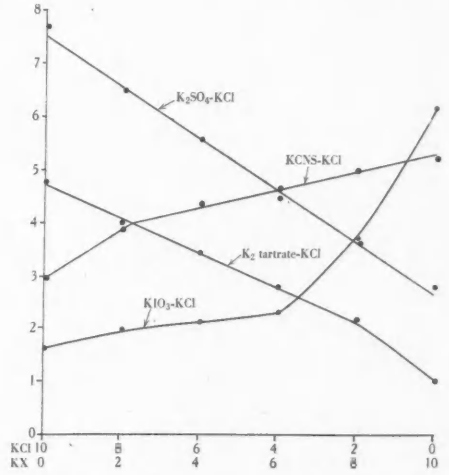

Fig. 3. Variation in the rate of penetration of K into the erythrocyte in mixtures of isotonic solutions of KCl and the K salts of different acids whose anions are referred to as X. The curves are not comparable one with another so far as the rates of penetration are concerned. Ordinates: rate of penetration of K in arbitrary units. Abscissae: proportions of KCl and KX solutions in the mixtures.

Cl-SO_4, Cl-tartrate and Cl-CNS mixtures do show a fair approximation to linearity; the Cl-IO_3 curve, on the other hand, obviously does not, indicating that the action of the IO_3 is definitely on the membrane itself. This inference is borne out by the observation that cells suspended in isotonic $NaIO_3$ for a few hours are permanently damaged, in that subsequent washing of cells so treated with isotonic NaCl causes haemolysis. The almost linear relation between the proportions of the two anions in the suspension medium and the permeability to K does not, of course, preclude an explanation of the effects of the anions on the basis of a direct action on the membrane.

Sodium permeability. Curves for the escape of Na from the same sample of cat erythrocytes in isotonic solutions of K salts of different univalent acids are shown in Fig. 4. Here a striking difference between K and Na permeabilities is observed, anions at the hydrating end of the lyotropic series, e.g. CNS and I, causing an almost complete inhibition of Na permeability; acetate which retarded K permeability in comparison with Cl causes an acceleration of Na permeability. The behaviour of IO_3 is irregular and conforms with the results on K permeability; NO_3 and Br, which accelerated K permeability as compared with Cl, retard Na permeability.

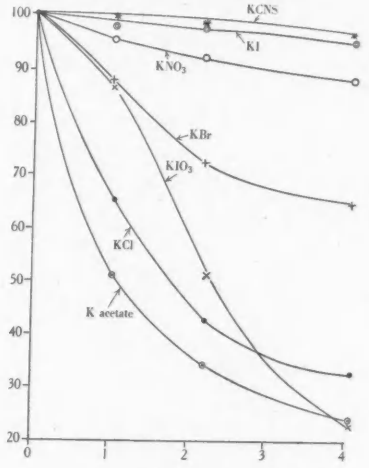

Fig. 4.

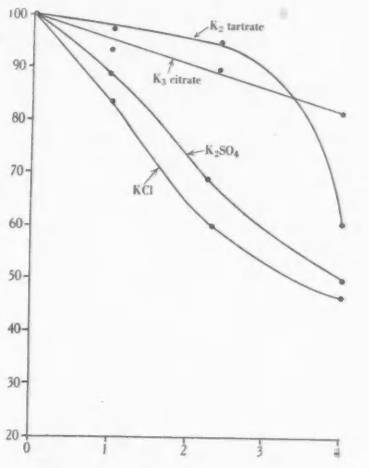

Fig. 5.

Fig. 4. Decrease in the Na content of the cat erythrocyte when suspended in isotonic solutions of the K salts of various acids. Ordinates: percentage of the original content. Abscissae: time in hours.

Fig. 5. Decrease in the Na content of the cat erythrocyte when suspended in isotonic solutions of the K salts of various acids. Ordinates: percentage of the original content. Abscissae: time in hours.

In Fig. 5 representative curves for the escape of Na in solutions of isotonic KSO_4, tartrate and citrate are shown compared with the escape in isotonic KCl solution. It is seen that the rate of escape in Cl solution is greatest, whilst the behaviour of tartrate is irregular in that a rapid acceleration of the rate of escape of Na occurs after the second hour. The SO_4, tartrate and citrate anions penetrate the erythrocyte membrane very slowly in comparison with the univalent anions (it is possible that citrate does not penetrate at all), so that a suspension of cells in the isotonic K salts of these acids is found to be acid, due probably to the exchange of OH ions in the medium with Cl and HCO_3 ions in the cells; as SO_4, tartrate etc. diffuse into the cell the reaction of the suspension medium becomes less acid. Since it has been shown earlier [Davson, 1940] that the permeability of the cat erythrocyte to Na is very sensitive to the *p*H of the medium, it was thought that the acidity caused by these ionic exchanges might retard Na permeability, so that it might quite possibly be that SO_4, for example, would actually accelerate the rate of escape of Na as compared with Cl. To test

this, cells were suspended in isotonic Na_2SO_4 solution for 3 hr., by which time the complete exchange of anions had taken place, and the reaction of the supernatant fluid was *p*H 7·4. The cells were then centrifuged down and re-suspended in isotonic K_2SO_4 solution, and the rate of loss of Na was compared with that from cells similarly treated with first Na and then KCl. The results are shown in Fig. 6, and it is clear that in these circumstances SO_4 has a pronounced accelerating action. This effect may, of course, be due to the necessity for SO_4 to act on the inside of the cell, quite apart from the *p*H changes; whatever may be the cause, it is clear that if SO_4 and Cl are allowed to act under comparable conditions, the rate of escape of sodium is greater in the Na solution. It seems quite possible that the acceleration of Na escape with time in tartrate solution is due to the slow diffusibility of tartrate ions into the cell; the regular behaviour of citrate, on the other hand, may be due to the complete indiffusibility of this ion through the erythrocyte membrane.

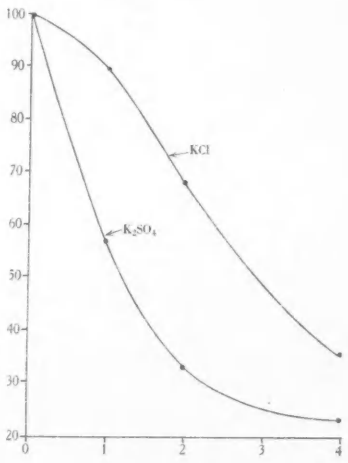

Fig. 6.

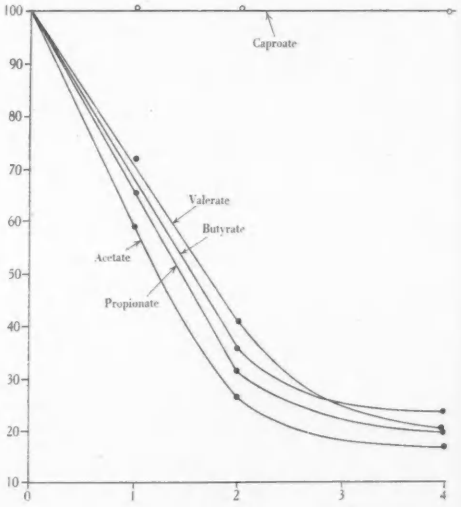

Fig. 7.

Fig. 6. Decrease in the Na content of the cat erythrocyte when suspended in isotonic KCl and K_2SO_4 solutions after a preliminary treatment with NaCl and Na_2SO_4 solutions. Ordinates: percentage of the original content. Abscissae: time in hours.

Fig. 7. Decrease in the Na content of the cat erythrocyte when suspended in isotonic solutions of the K salts of straight-chain fatty acids. Ordinates: percentage of the original content. Abscissae: time in hours.

The effect of the series of straight-chain fatty acids is shown in Fig. 7; increasing the length of the hydrocarbon chain causes a small decrease in permeability until we pass from valeric to hexoic acids, in which case complete inhibition of Na permeability is observed.[1] Höber [1936], in attempting to explain

[1] An isotonic solution of the K salt of octoic acid, was found to be haemolytic; however, it was found that a mixture of isotonic KCl and K octoate in equal proportions was not haemolytic and inhibited Na permeability.

the haemolytic effect of Na salts of the higher fatty acids, suggests that the haemolysis is due to hypotonicity owing to the formation of micelles; on this basis it may be argued that the inhibition of Na permeability by hexoate is due to hypotonicity, which also inhibits Na permeability. That this explanation is untrue is shown by measuring the permeability in 0·20 *M* K hexoate, in which case inhibition is also found; furthermore the 0·165 *M* solution may be diluted to at least 0·13 *M* without producing any haemolysis.

The effect of varying the proportions of CNS and Cl and of I and Cl in the suspension medium on Na permeability is shown in Fig. 8, and it is clear that

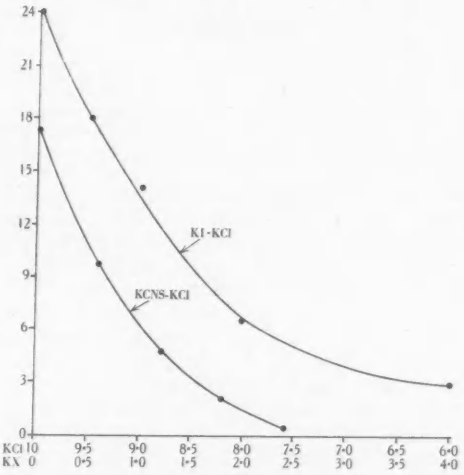

Fig. 8. Variation in the rate of penetration of Na from the erythrocyte in mixtures of isotonic solutions of KCl and KI and of KCl and KCNS. Ordinates: rate of escape of Na as percentage of the original content per hour. Abscissae: proportions of KCl and KX in the mixtures.

the amount of CNS which must be present to produce complete inhibition is of the order of 0·045 *M*. The curves do not represent experiments on the same blood but comparative experiments under these conditions indicate that CNS is much more effective than I in inhibiting Na permeability. For convenience the effects of mixtures ranging from pure KCl to six parts of the latter to four of KCNS and KI only are shown; if the curves were extended to show the effects of richer mixtures the complete absence of any semblance to linearity in the relation between permeability and proportions of Cl and CNS and of Cl and I would be more evident.

Discussion

The results described in this paper indicate a dual behaviour of anions according as K or Na permeability is being studied. The hydrating anions, e.g. CNS and I, cause an acceleration of K permeability and a retardation of Na permeability, and the hydrating anions, e.g. SO_4, have the reverse effects in the two cases. This duality has already been observed in regard to the effects of alkaline earths, tonicity, ageing and narcotics [Davson, 1940], and it would seem that there is some fundamental difference between the two modes of penetration.

It has been suggested earlier that the influence of anions on permeability may at least in part be due to diffusion potentials; qualitatively the experiments on K permeability would appear to bear out this viewpoint. It must be realized, of course, that the equation of Nernst involves mobilities of the ions, and the assumption made in this discussion is that the mobilities are proportional to the penetrations of the ions through the membrane: if the penetration of K occurs through a pore in the membrane and if the number of pores available for K diffusion is very small in comparison with those available for anion penetration then it would be quite possible that the actual mobility of the K ion through the membrane is of the same order of magnitude as that of anions, in fact it may even be greater than that of SO_4, for instance, whilst the relatively low penetration would be due to the limited number of pores available for K diffusion. If this were the case it seems possible that the simple equation for the diffusion of electrolytes in aqueous solution is not applicable without modification to the more complicated problem of permeability.

So far as Na permeability is concerned, the diffusion potential factor, if it is present, is obviously obscured by some other effect; the extraordinary inhibiting effects of CNS, I and hexoate are strongly reminiscent of the action of narcotic substances described earlier. With these substances it was found that Na permeability may be completely inhibited whilst under the same conditions K permeability was only accelerated. It has also been found (unpublished) that surface-active substances such as Na oleate will in very low concentrations (1 part in 10,000 in this case) also inhibit Na permeability, and it is interesting that CNS and I ions exert their lyotropic effects in virtue of their adsorbabilities at a protein-water interface. Again, the sudden transition in behaviour in passing from valeric to hexoic acids is suggestive that the anion is exerting its influence in virtue of its adsorbability at the membrane-aqueous interface, in fact the work of Schulman & Rideal [1937] on the penetration of mixed lipoid-protein films would suggest that at a certain stage in a homologous series of fatty acids, the acid will actually penetrate the film, as opposed to a head-to-head adsorption with the hydrocarbon end of the acid remaining in the water phase. It is interesting, in this connexion, that Schulman & Rideal have shown that the strongly surface-active substance, saponin, will not penetrate a lipoid-protein film, and unpublished work of Davson has shown that saponin has no influence on Na or K permeability, nor yet does it inhibit the penetration of glycerol into the rabbit erythrocyte, although this permeability process is affected by narcotic substances [Anselmino & Hoenig, 1930; Jacobs & Parpart, 1937]. Na permeability may thus be inhibited by narcotic substances such as amyl alcohol, ether, urethanes etc., by soaps and by adsorbable anions such as CNS and I. If there is a factor in common with all of these substances it is apparently not their ability to hydrate colloids, since ether is just as effective as CNS. The full explanation of the phenomena must obviously await further quantitative experimental work on the influence of narcotic substances and of other anions. The results of this paper show beyond doubt that the behaviour of the erythrocyte membrane, so far as ionic permeability is concerned, is not related in any simple manner, if at all, to hydration phenomena.

Summary

The influence of the lyotropic series of anions on Na and K permeability in the cat erythrocyte has been studied. Anions at the dehydrating end of the series, e.g. SO_4, retard K permeability and apparently accelerate Na permeability.

Anions at the hydrating end of the series, e.g. CNS, have the reverse effects in the two instances. If the permeability to Na is measured in isotonic solutions of the K salts of the homologous series of straight-chain fatty acids, a small decrease in permeability occurs with each step from acetate to valerate; on passing from valerate to hexoate permeability is completely inhibited. It is argued that the results show that the effects of anions on permeability phenomena are not predictable from a knowledge of their behaviour in respect to the hydration of colloidal systems.

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III. INHIBITION BY FLUORIDE OF GLUCOSE BREAKDOWN IN TUMOUR TISSUE AND RETINA EXTRACTS

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(*Received 9 March 1940*)

THE process by which glucose is broken down to lactic acid in animal tissue is not yet known in detail. The possibility still remains that the production of lactic acid proceeds, in some tissues, without the intermediate formation of phosphate compounds [Ashford & Holmes, 1929; Needham & Lehmann, 1937]. The following estimations of inorganic phosphate in tumour brei during glycolysis may therefore be of interest, although it cannot be claimed that they afford definite proof of a "non-phosphorylating" glucose breakdown in this tissue.

Fluoride was used in the ordinary way to prevent the formation of lactic acid and allow of the accumulation of phosphate esters. In the earlier experiments concentrations as high as $N/20$ or $N/40$ NaF were used, but experiments using $N/100$ or $N/200$ NaF were later carried out and gave exactly similar results. All experiments were done in the Warburg apparatus at 37°. A control manometer, in which no fluoride was present, was frequently included in order to determine the amount of lactic acid that could be made by the tissue in the conditions of the experiment. This gave a guarantee that the tissue was capable of active glycolysis and gave some indication of the amount of hexose ester that might be expected to accumulate in the presence of NaF.

Some experiments were carried out in N_2 and others in 95% N_2+5% CO_2, the latter with the usual $NaHCO_3$ buffer. A single tumour was used for each experiment, obviously necrotic tissue being removed and 0·25 or 0·3 g. of tissue taken for each Warburg cup after chopping and mixing.

The figures given in Table 1 show that no disappearance of inorganic phosphate occurred with the tumours named in column 1 under anaerobic conditions

Table 1

	Inorganic P (mg.)			Lactic acid (mg.)	
Tissue and gas phase	Resting value	With glucose and NaF	No glucose no NaF	Resting value	With glucose no NaF
Tumour 113; N_2+CO_2	0·68	0·67	—	0·38	1·12
Crocker tumour; N_2+CO_2	1·82	1·82	1·95	0·70	2·0
Crocker tumour; N_2+CO_2	1·72	1·74	1·86	0·9	2·64
Crocker tumour; N_2	1·39	1·54	1·58	1·32	3·89
Crocker tumour; N_2	1·60	1·51	1·62	0·97	3·3
Tumour 113; N_2+CO_2	0·60	0·65	—	0·31	1·51
Crocker tumour; N_2	1·20	1·14	1·26	—	2·58

in the presence of glucose and NaF. Considerable amounts of lactic acid were formed by the control samples with NaF omitted (Table 1, columns 5 and 6), which suggests that, if phosphate esters were formed, they should have been present in large enough amounts for the disappearance of inorganic phosphate

to be easily detectable. These results are in agreement with those of Ashford & Holmes [1929], who found no evidence of any ester formation from glucose in brain.

The possibility had to be considered that glucose might have been phosphorylated by organic phosphate compounds which were not rephosphorylated under the conditions of the experiment and thus did not cause disappearance of inorganic phosphate. In order to test this as far as possible, control samples were subjected to the same treatment as the others, but were kept in medium containing neither glucose nor NaF. It might be expected that under these conditions and in an experiment lasting 2–3 hr. such organic compounds would break down and an increase of inorganic phosphate would be detected. It can be seen, however (Table 1, column 4), that such increases are always very small. If organic compounds exist in the tumour tissue which are capable of phosphorylating glucose they must be compounds which are not broken down by the tissue phosphatases. (The phosphate estimations were kindly carried out by Dr Lehmann.)

Some experiments were also carried out to compare the effects of very dilute NaF, *N*/1000, on the breakdown of glucose and of hexosediphosphate. The experiments were done in 0·26% $NaHCO_3$ buffer in presence of 95% N_2 + 5% CO_2 and the production of acid was followed on the manometer. The inhibition of acid production was greater in the case of glucose, but when estimations of lactic acid were made at the end of the experiment it was found that all the acid formed from glucose was lactic acid whereas a considerable amount of the acid formed from hexosediphosphate was not lactic acid. It has been shown by Needham & Lehmann [1937] that in chick embryo even dilute NaF inhibits the change of phosphoglyceric acid into phosphopyruvic acid. The unknown acid, which was recorded in the manometer readings but could not be estimated as lactic acid, was, therefore, probably phosphoglyceric acid.

The figures are given in Table 2. They represent the total acid and lactic acid made from glucose and hexosediphosphate during the experiment, the resting value and the small amount of acid formed by the tissue without added

Table 2. *Lactic acid formed from glucose or from hexosediphosphate*

Amounts expressed in mg.

Exp.	Additions	Glucose	Glucose + NaF	H.D.P.	H.D.P. + NaF
(1)	Lactic acid found	0·30	0·21	1·1	**0·30**
	Acid calculated from CO_2	0·34	0·21	1·0	**0·65**
(2)	Lactic acid found	1·0	0·71	—	**0·23**
	Acid calculated from CO_2	0·95	0·83	0·98	**0·80**
(3)	Lactic acid found	0·9	0·57	0·6	**0·20**
	Acid calculated from CO_2	0·76	0·40	0·5	**0·43**
(4)	Lactic acid found	0·63	0·37	0·72	**0·34**
	Acid calculated from CO_2	0·45	0·33	0·78	**0·53**
(5)	Lactic acid found	0·38	0·20	0·85	**0·36**
	Acid calculated from CO_2	0·36	0·16	0·82	**0·60**
(6)	Lactic acid found	0·50	0·21	0·76	**0·20**
	Acid calculated from CO_2	0·38	0·23	0·68	**0·50**

substrate having been subtracted. The inhibition of lactic acid formation from hexosediphosphate is not complete and if the experiment is continued too long, i.e. after the production of acid as shown by the manometer readings has become very slow, this intermediate acid will not be found. No doubt after its further

formation has ceased the intermediate acid is slowly converted into lactic acid without significant change of *p*H or evolution of CO_2.

The description of hexosediphosphate glycolysis in tumour tissue is given by Boyland *et al.* [1937] and also by Holmes [1937; 1939]. Mouse tumour 113, which was used in the experiments just described, is exceptionally active in this respect. Cozymase and adenylic acid must, however, always be added as activators. These activators were tipped into the main part of the Warburg flask just before taking the initial manometer reading. The values of acid measured manometrically and lactic acid estimated chemically should therefore have been in good agreement and in the flask without NaF were so. Lactic acid formation from glucose, which did not require the addition of activators, must have proceeded during the setting up of the experiment and, as expected, the manometric estimation of lactic acid was usually a little lower than the chemical estimation. The contrast with the figures given in the last column of Table 2 is marked.

The figures are all fairly small, since one tumour of this type yields only small amounts of living tissue. It should be pointed out that the evolution of a small amount of CO_2 produces a large excursion on the manometer of the specially small (vol. 6–7 ml.) Warburg apparatus employed. The amount of lactic acid present was ample for duplicate estimations by the method of Friedemann *et al.* [1927].

One possible explanation of the figures given in Tables 1 and 2 is that an early stage of glucose breakdown is inhibited by NaF, so that intermediate compounds are not formed and therefore not detectable. It is perhaps not possible to know from experiments with NaF whether phosphorylation of glucose does, in fact, occur. If it does, the process of phosphorylation must differ from that of glycogen phosphorylation in its sensitivity to NaF, since this can be used to detect the intermediate products of glycogen breakdown.

Extracts of retinae [Kerly & Bourne, 1940; Süllman & Vos, 1939] can form lactic acid from both glucose and glycogen. Preliminary experiments with such extracts suggested that, even in this case, glucose glycolysis was inhibited by lower concentrations of NaF than was glycogen glycolysis. Furthermore, it could be shown with these extracts as well as with the tumour brei, that when lactic acid formation was inhibited by NaF all disappearance of inorganic phosphate was also inhibited. On the other hand the finding of Süllman, that inorganic phosphate disappears from the extracts in the presence of glucose and without the addition of NaF, was confirmed. In such extracts lactic acid is rapidly formed and inorganic phosphate disappears. The addition of *N*/200 NaF inhibited both these processes. The results are given in Table 3 and include an experiment in which zinc was added as inhibitor. It had been shown by Lehmann [1939] that zinc does not inhibit the formation of Cori ester (glucose-1-phosphate) from glycogen by muscle extract. It is obvious that, in this experiment, it inhibited the formation of any hexose ester from glucose.

In Table 3 are included two experiments with tumour tissue, which showed that when glycolysis was wholly or partially inhibited by dilute NaF, the glucose which was not broken down to lactic acid remained as glucose. It is therefore not surprising that there is no disappearance of inorganic phosphate in similar conditions (Table 1). It seems that the only conclusion that can be drawn from these experiments with tumour and retina in the presence of NaF is that the first stage of glucose breakdown is particularly sensitive to this inhibitor. The work of Ashford & Holmes [1929] and Ostern *et al.* [1939] included similar experiments with NaF: brain and liver tissues were used; no disappearance of

Table 3

Exp.	Retina extract	Glucose mg.	Inorganic P mg.	Lactic acid (calc. from manometer readings) mg.
1	Resting value	3·3	0·60	—
	No inhibitor	0·73	0·31	1·2
	N/2000 $ZnSO_4$	3·0	0·60	Nil
2	Resting value	2·72	0·34	—
	No inhibitor	0·74	0·0	1·2
	N/400 NaF	2·4	0·30	0·30
3	Resting value	4·5	—	—
	No inhibitor	0·74	—	2·1
	N/200 NaF	4·1	—	Nil
4	Resting value	—	0·51	—
	No inhibitor	—	0·23	1·75
	N/120 NaF	—	0·50	Nil
	Crocker tumour brei			
5	Resting value	2·3	—	—
	No inhibitor	1·36	—	0·81
	N/400 NaF	1·91	—	0·41
6	Resting value	2·09	—	—
	No inhibitor	1·03	—	1·2
	N/120 NaF	2·03	—	0·36

inorganic phosphate occurred in the presence of glucose and NaF. Here again, the explanation may be that the whole process of glucose breakdown was inhibited.

Süllman & Vos [1939] do not make any suggestion as to the nature of the phosphate compounds formed when inorganic phosphate disappears during glycolysis in retinal extracts without NaF. This matter is now under investigation. It is almost certain that some part of this phosphate is used in phosphorylating the adenylic acid which has to be added to the extracts as activator[1] but the fate of the rest of it is as yet unknown.

Summary

1. Experiments with tissue from the Crocker tumour and mouse tumour 113 showed that no disappearance of inorganic phosphate occurred in the presence of glucose and NaF. No evidence could be obtained of phosphate ester formation from glucose.

2. It was found that the disappearance of glucose in these preparations was inhibited by the addition of NaF.

3. It was thus plain that the presence of NaF inhibited glucose breakdown at the first stage and prevented the formation of any intermediate compounds.

4. Experiments with extracts of pigs' retinae gave similar results. The disappearance of inorganic phosphate which occurs during glucose glycolysis was inhibited by the presence of NaF, as was also the disappearance of the glucose itself.

5. Dilute NaF (*N*/1000) inhibited the formation of lactic acid from hexosediphosphate by tumour tissue and caused the accumulation of some acid other

[1] On one occasion this occurred even in the presence of NaF. It led to disappearance of phosphate which was not accompanied by any disappearance of glucose. The phosphate could all be recovered from the adenyl pyrophosphate fraction.

than lactic acid. This did not occur when glucose was used instead of hexose-diphosphate.

I have great pleasure in thanking the Imperial Cancer Fund for a personal grant, and Dr H. Lehmann for carrying out the phosphate estimations.

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112. SEROMUCOID AND THE BOUND CARBOHYDRATE OF THE SERUM PROTEINS

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Received 29 April 1940

THE past few years have witnessed a renewal of interest in the carbohydrate groups of proteins, particularly those of serum. Earlier investigators had claimed that serum albumin and globulin afforded a positive Molisch reaction, but the complex polysaccharide was isolated for the first time from each of these proteins by Rimington [1929; 1931]. Glucosamine and mannose were detected as components of this complex [Rimington, 1929] and the presence of galactose also has been rendered probable [Sörensen & Haugaard, 1933; see also Dische, 1928; Bierry, 1929]. For a more detailed historical presentation see Rimington [1933]. Although, in these experiments, evidence was presented that the carbohydrate groups were firmly united to the protein, it was shown later by Sörensen & Haugaard [1933] that, by a suitable fractionation procedure, there could be produced from crystalline serum albumin a series of crystalline sub-fractions differing widely in their carbohydrate contents and other properties, those most readily soluble containing approximately 50 times as much carbohydrate as those least soluble. This finding was regarded as further evidence for Sörensen's theory of the nature of soluble proteins as associations of mutually interacting component systems [Sörensen, 1930].

More recently still, Hewitt [1936; 1937; 1938; 1939] has obtained from serum albumin both carbohydrate-rich and practically carbohydrate-free materials. His interpretation, however, is that the pure albumin ("crystalbumin") is devoid of carbohydrate but that, as usually prepared, it contains quantities of two other proteins, "seroglycoid" and "globoglycoid", both of which contain carbohydrates. His view thus differs sharply from that of Sörensen. The nature of the association of crystalbumin with the other two proteins is not precisely defined, but Hewitt seems to regard it as a simple admixture. He advances reasons for considering "seroglycoid" to be different from the "seromucoid" of earlier investigators.

Seromucoid appears to have been first discovered by Freund [1892] who, however, overlooked the presence of nitrogen in his preparation which he named "Tiergummi". It was later investigated by Zanetti [1897; 1903], who gave it the name "seromucoid", considering it to be similar to ovomucoid. Bywaters [1909] studied the protein more thoroughly, obtaining it in the same way as his predecessors from the filtrate from the heat-coagulated serum albumin and globulin by precipitation with alcohol. The yield of purified material was 0·3–0·9 g./l. of blood, but varied according to the nutritional state of the animals. The protein contained 25% of carbohydrate and pentabenzoylglucosamine was obtained from the acid hydrolysate. Ozaki [1936] has also described the preparation of seromucoid by an essentially similar method and claims that it contains 13·4% of reducing sugars, calculated as glucose after hydrolysis.

I have recently been able to resume a study of seromucoid begun many years ago. The present paper deals with the isolation and properties of this constituent from ox serum, and with methods for determining the total hexose and glucosamine contents of proteins. The question of the relation of the seromucoid of horse serum to other constituents of the albumin fraction, with their specific immunological relations, is considered in the following paper with Dr M. Van den Ende.

Source of materials

In the present investigation, ox blood was employed, and as it was necessary to work with large amounts of material, the preliminary operations were carried out for me by Messrs Boots Pure Drug Co. of Nottingham. To them and to the biochemist in charge of the operations, Mr H. Calam, I am very greatly indebted for their generous assistance. The method employed was as follows.

Ox blood, collected and defibrinated at the slaughter-house, was mixed with an equal volume of 0·9 % NaCl containing "Chinosol" to prevent putrefaction. After removal of the erythrocytes, the serum was acidified with acetic acid to about *p*H 4·7 and then heated to coagulate the albumin and globulin, which were filtered off after very careful adjustment of the *p*H to ensure maximum precipitation.

The clear filtrate was concentrated *in vacuo* at 50° to a small volume and treated with 10 vol. of alcohol. The precipitate, containing the crude seromucoid and much inorganic material, was washed with alcohol and ether and dried *in vacuo*. In all, approximately 1 kg. of this material was prepared (with a mean yield of 3 g./l. of blood). It was found, however, to contain over 92 % of incombustible ash. It was a light brown powder, soluble in water and giving strong biuret and Molisch reactions; 2 % of trichloroacetic acid produced only a faint opalescence.

Purification and fractionation of the crude seromucoid

Concentrated solutions of the crude seromucoid, when boiled at *p*H 4·7, developed only a faint opalescence, so that coagulable protein was virtually absent. The ratio of total carbohydrate[1] to total N before dialysis was of the order of 1·4 : 1 and was reduced only slightly by prolonged dialysis in collodion sacs. 500 g. crude seromucoid were stirred mechanically with 2 l. of water and some insoluble material removed by filtration. The solution was then dialysed against running tap water for 8 days, thymol being added as a preservative. At the end of this time, some dark brown material had separated in the solution and as an encrustation upon the dialysing membrane; it was removed by centrifuging. The solution now had a carbohydrate/N ratio of 1·14 : 1.

Addition of 2·75 vol. of alcohol produced a bulky precipitate which was collected at the centrifuge. When stirred with water again this material did not completely redissolve; the residue gave a red colour with H_2SO_4, and with acetic anhydride and chloroform a typical Liebermann-Burchard reaction, indicating the presence of sterols; the biuret reaction was also positive. Material of this description was invariably present in crude seromucoid preparations and was very difficult to remove. Each time a clear mucoid solution was precipitated with alcohol, there remained, on attempting to redissolve it, a further quantity

[1] Expressed as glucosamine-galactose-mannose (see later). Ratios of carbohydrate to total N are preferable to calculations as percentages, since in the latter case the N content of the protein has to be assumed.

of this lipin-containing material, but by subjecting the crude protein to prolonged and repeated treatments, with absolute alcohol and ether, the lipin could be almost completely removed. It is difficult to decide whether or not seromucoid is actually combined with these sterol and lipin bodies in serum; the presence of carbohydrate in the insoluble residues from crude material may be due merely to mechanical co-precipitation, and in this connexion one may recall the separation of similar material with the albumin crystals from natural serum not freed from lipins. Purified seromucoid, as finally obtained, is not rendered insoluble by contact with alcohol; in this respect, as in its non-coagulability on heating, it resembles other mucoids such as ovomucoid.

Fractional precipitation with alcohol of seromucoid freed from lipins was carried out by adding alcohol in successive stages to the solution and removing the precipitates formed. These were redissolved and analysed with the results shown in Table 1.

Table 1

Fraction	Vol. of alcohol added	Total carbohydrate/total N	Remarks
Original	—	1·14	—
Precipitate A	1	0·95	Bulk of material
Precipitate B	1·5	1·72	—
Precipitate C	4	4·40	Very small quantity
Do.	— *	4·43	—

* After dialysis.

The bulk of the material was represented by fraction A. This was redissolved in 400 ml. of water and an equal volume of alcohol added, thereby producing a fairly abundant precipitate P and a filtrate F; the treatment of each will be separately described.

The precipitate P was redissolved in 250 ml. water with the addition of a few drops of alkali (to *p*H 6·5), 75 ml. of alcohol were added (to 23 %), the reaction adjusted to *p*H 4·0 and the small precipitate *a* removed; a further 25 ml. of alcohol (to 29 %) brought down precipitate *b*, and so on, as shown in Table 2, until the filtrate finally gave no further test for protein.

Table 2

Fraction	Alcohol added % conc.	Total carbohydrate/total N	Remarks
Original	—	0·82	—
Precipitate *a*	23	0·68	Small precipitate; requires alkali for resolution
Precipitate *b*	29	0·79	Combined and reprecipitated affording main precipitation 0·79, called M
Precipitate *c*	37·5	0·79	
Precipitate *d*	50	0·80	
Precipitate *e*	66	1·25	Small precipitate

It will be seen from Table 2 that, with the exception of the two end fractions *a* and *e*, the material had a constant carbohydrate/N ratio, corresponding to about 10·7 % of glucosamino-dihexose in the protein. The three fractions, *b*, *c* and *d*, comprising the bulk of the material, were therefore combined and once more reprecipitated and named fraction M.

Returning now to the filtrate F from 50 % alcohol mentioned above, when the alcohol concentration was further raised to 66 %, there separated a small quantity of material with carbohydrate/N ratio of 0·91, and at still higher

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alcohol concentration (80 %) the remaining material, very small in amount, was recovered and had a ratio of 3·0. This material, designated "G", was used later when determining hexose/glucosamine ratios (see that section). It is thus clear that, accompanying the main protein, there was a small quantity of material which required a relatively high concentration of alcohol for precipitation, and which was probably a mixture of peptone-like bodies. The substances present in this fraction were comparatively rich in carbohydrate, and may well have been derived from the seromucoid itself by decomposition of a portion of the latter during the process of isolation, though the possibility that they actually exist in native serum cannot be excluded.

Analysis of the different fractions

Whilst the greatest interest naturally attached to the homogeneous, main fraction M, it was considered instructive to analyse in detail also the fractions *e* and C, having carbohydrate/*N* ratios of 1·25 and 4·43 respectively, and also a small preparation A 2 from another batch, prepared in a slightly different way, with a ratio 1·26. The materials were dehydrated by alcohol and ether, and then dried to constant weight *in vacuo*. The ash contents were determined and the other percentages calculated upon an ash-free basis. The results are assembled in Table 3.

Table 3. *Analytical data for certain fractions*

Fraction	Ash %	C %	H %	N %	S %	Acetyl	Carbohydrate as glucosamino-dihexose	Total carbohydrate/total N
Main seromucoid M	0·37	50·32	6·89	13·60	1·59	Present	10·7	0·79
Slightly more soluble fraction *e*	0·46	50·76	7·02	12·52	0·94	,,	15·6	1·25
Differently prepared A 2	0·69	51·73	6·99	13·31	0·64	,,	16·8	1·26
Very soluble end-fraction C	4·35	48·81	6·71	9·67	0·44	2·48	42·8	4·43

On fraction C it was possible to make a reasonably accurate acetyl determination and the figure of 2·48 % obtained is suggestively near to that of 2·65 %, which would be required by the assumption that glucosamine is present in the carbohydrate complex as the *N*-acetyl derivative.

Determinations of optical rotations and of contents of certain amino-acids are reproduced in Table 4. The figures given are the means of several concordant determinations. Tyrosine and tryptophan were determined by the Folin & Marenzi [1929] method.

Table 4

Fraction	$[\alpha]_{5461}$	Cystine	Tyrosine	Tryptophan	Carbohydrate content
M	−53·99°	2·28	2·98	1·03	10·7
e	−55·13°	2·06	2·63	0·98	15·6
C	—	—	1·08	1·04	42·8

The determination of total hexose in glycoproteins

For the determination of non-amino sugars in proteins, the colorimetric method employing orcinol, originally put forward by Tillmans & Philippi [1929], has been widely adopted. It was used by the writer [Rimington, 1931] in his

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early work upon the carbohydrate complex of serum albumin and globulin and in a slightly modified form by Lustig & Haas [1931] in their sub-fractionation experiments. Sörensen & Haugaard [1933] made a careful study of the method and, on the basis of the time curves of colour development given by various sugars, used it to identify the hexose constituents present in a number of proteins. In connexion with later experiments on lactalbumin, Sörensen [1936] showed that the data given by the time curves are not entirely reliable owing to interference, particularly by tryptophan. She now concludes that both mannose and galactose are present in this protein, and states that casein may also contain another hexose in addition to galactose. Hewitt [1934] made the important observation when using the orcinol method that the solutions must be kept shielded from the light. The Japanese workers have employed both this and the indole colorimetric method for the differentiation of hexoses.

For the present investigation, a closer study of the conditions affecting colour development has been made. The points requiring emphasis are as follows: In order to obtain reproducible results the temperature of the water bath should be controlled at $80 \pm 0{\cdot}5°$, and the time of immersion of the solutions accurately timed (20 min. is a convenient time). The concentration of the H_2SO_4 should be 60 % by volume or 68·5 % by weight (sp. gr. 1·592 at 20°), and the acid orcinol solution should be stored in the dark and rejected if it acquires a pinkish brown tint. The colour developed by 1 ml. of sugar solution + 2·5 ml. of orcinol (1·6 % in 30 % by volume H_2SO_4) + 15 ml. of 60 % by volume H_2SO_4, heated at 80° for 20 min., then cooled by immersion in tap water, was examined in a Hilger Spekker absorptiometer. Using the green Ilford Spectrum filter No. 604 (max. transmission 5200 Å.) mannose, galactose and glucose give identical readings under these conditions; with the blue filter No. 602 (4700 Å.) there is some divergence, mannose affording somewhat higher readings than galactose [compare Lustig & Langer, 1931].

Throughout these investigations each unknown solution was read against both the green and blue filters and standard reference curves, one for each filter, derived from a mixture of equal quantities of mannose and galactose, were employed, but no significant deviation from this ratio was found in any of the fractions analysed. Thus it may be concluded that in seromucoid, as in the serum albumin fractions investigated later, mannose and galactose are present in the polysaccharide complex in equimolecular proportions. As is reported below, the ratio of non-amino sugar to glucosamine was similarly constant throughout the series at 2 : 1. Thus the polysaccharide unit in seromucoid consists of glucosamine-galactose-mannose, and all figures for total carbohydrate have been

Table 5. *Distribution of bound carbohydrates in 500 ml. normal horse serum*

Fraction	Hexose by orcinol method (g.)	Ratio: Hexose/total N
Total serum proteins	1·116	0·339
Globulin	0·531	0·333
Crystalline albumin	0·170	0·100
Insoluble lipoid material accompanying crystalline albumin	0·019	—
66 % $(NH_4)_2SO_4$ fraction	0·220	0·315
Insoluble material accompanying above	0·023	—
75 % $(NH_4)_2SO_4$ fraction	0·014	0·538
Insoluble material accompanying above	0·001	—
100 % $(NH_4)_2SO_4$ fraction	0·014	1·370
Total	0·992	

calculated and expressed accordingly. The total quantity of bound carbohydrate in normal horse serum is approximately 0·2%, and a typical determination of its distribution between the individual fractions is shown in Table 5. No sub-fractionation of the globulins was attempted; Lustig and his associates [1931] have provided abundant data concerning these groups and the present figures for the albumin fraction confirm, in general, his findings for his albumins I, II and III.

The determination of glucosamine

For the colorimetric determination of glucosamine in simple solutions, the procedure of Elson & Morgan [1933] is satisfactory. Difficulties have been encountered, however, in attempting to apply this method to the analysis of glycoproteins. When my work was begun, the only methods available were those of Masamune & Nagazumi [1937] and Nilsson [1936]. The conditions for hydrolysis, etc. advocated in these two papers differ widely; Nilsson used 3 hr. hydrolysis in *N* HCl, and the Japanese workers 5·5*N* acid for 5 hr., followed by a rather elaborate procedure for the removal of excess Cl ions. Again, the compositions of the alkaline acetylacetone solution are different in the two cases. A trial of both methods showed that neither was completely satisfactory, but the following procedure has proved both convenient and reliable.

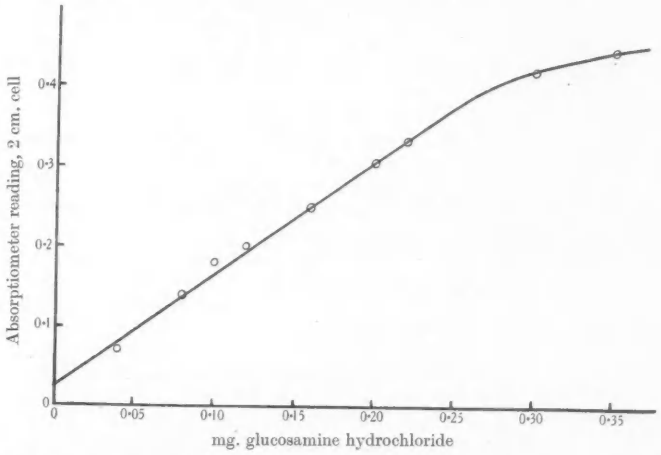

Fig. 1. Proportionality between colour developed and amount of glucosamine present.

10–20 mg. of protein, depending upon the glucosamine content, are placed in a small flask and refluxed for 4 hr. with 5–10 ml. of *N* HCl and 2 drops of capryl alcohol. This procedure is sufficient to liberate all the glucosamine, as is illustrated by Fig. 2. The equivalent quantity of *N* NaOH is then added to neutralize the solution and the deposit of humin removed at the centrifuge. 4 ml. of the hydrolysate, containing about 0·2 mg. of glucosamine, are used for the determination. The quantity of NaCl present does not interfere with colour development. 2 ml. of Elson & Morgan's acetylacetone reagent (1 ml. freshly dissolved in 50 ml. of half *N* Na_2CO_3) are added and the tube, provided with a glass bulb to prevent evaporation, immersed for 20 min. in a boiling water bath.

After cooling, 5 ml. of alcohol and then 2 ml. of Ehrlich's reagent (0·8 g. repeatedly recrystallized *p*-dimethylaminobenzaldehyde dissolved in 30 ml. alcohol + 30 ml. conc. HCl) are added, and 45 min. are allowed for colour development. 2 ml. of alcohol are then added to adjust the volume to 15 ml. and the colour intensity is measured in the Hilger Spekker absorptiometer, using the Ilford green filter No. 604. A blank tube containing no protein solution is run at the same time. By reference to a standardization curve, the amount of glucosamine is read off. This curve (Fig. 1) was obtained with pure glucosamine hydrochloride, and it will be seen that up to about 0·25 mg. of glucosamine the relation between extinction and concentration is linear.

Since this technique was evolved, Sörensen [1938] has published a study of the problem, but recommends a stronger acid for hydrolysing the protein. In other respects her method does not differ greatly from that here described.

The rate of liberation of glucosamine during hydrolysis and determination of the hexose/glucosamine ratio

The progress of the liberation of free glucosamine from a glycoprotein was followed, using a preparation of ox pseudoglobulin.

2 g. of protein were refluxed with 100 ml. of *N* HCl in a tared flask and 5 ml. samples withdrawn at intervals. A few drops of capryl alcohol were present to prevent foaming. After neutralization of the sample, 1 ml. was used for non-amino sugar determination by the orcinol method and 4 ml. for glucosamine as described above. Total N was determined upon 5 ml.

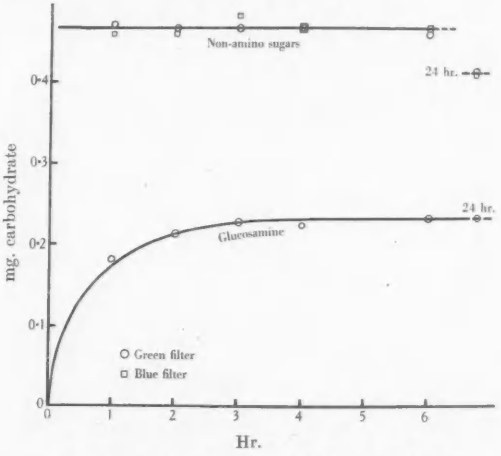

Fig. 2. Hydrolysis of ox pseudoglobulin by *N* HCl.

The results are shown on a graph in Fig. 2. Liberation of glucosamine is complete within 4 hr., the value then remaining unchanged up to 24 hr. The hexoses are slowly destroyed by the boiling acid, so that the 24-hr. value for these is appreciably lower than the earlier determinations. The mean of these gave 16·8 mg./100 g., and the correspondence between the values using the blue and green filters indicated equimolecular proportions of galactose and mannose.

The glucosamine was 8·43 mg./100 g., hence the ratio galactose-mannose : glucosamine = 1·99 : 1.

Several of the individual seromucoid fractions were analysed in this way, making the determinations after 4 hr. hydrolysis; 10–20 mg. of protein sufficed in each case, and all colorimetric determinations were made in duplicate. The results are shown in Table 6.

Table 6

Fraction	Total carbohydrate/total N	Hexose	Glucosamine	Hexose/glucosamine
Main seromucoid M	0·79	0·116	0·0561	2·07
Small fraction G separated at 66 % alcohol concentration	3·0	0·057	0·025	2·28
Very soluble peptone-like fraction C	4·43	0·123	0·0606	2·03

It is thus clear that in all fractions 2 mol. of hexose are associated with 1 of glucosamine.

Discussion

Since the original claims of Zanetti [1903] and Bywaters [1909] that there exists in serum a non-coagulable protein which is rich in carbohydrate, considerable doubt has been felt about the status of the so-called "seromucoid". The method of preparation employed by these workers involved drastic treatment, and no satisfactory evidence other than elementary analyses was offered to indicate the homogeneity of the materials obtained.

I have confirmed the fact that a protein, rich in carbohydrate and not coagulated by heating, is left behind in serum after the separation of the coagulable proteins. By employing more gentle methods for its preparation and purification, the greater part of this material has been obtained in an apparently homogeneous form, since fractional precipitation with increasing quantities of alcohol yields products with a constant carbohydrate/N ratio of about 0·79. If the term seromucoid is to be given a definite significance, it seems rational to limit its use to this homogeneous fraction. There are also present, however, in the filtrate from the heat-coagulated proteins, other substances less readily precipitated by alcohol and probably of peptone-like nature, in which carbohydrate may account for nearly 50 % of the whole molecule. These materials have not been purified, but like the homogeneous seromucoid, they are incoagulable by heat, and their carbohydrate component appears to be the same. It seems probable that they may be split-products derived from the native seromucoid during the course of its isolation.

The purification of seromucoid is further rendered difficult by the presence in the crude filtrates from serum of lipin-protein complexes, which give the reactions for sterols and also appear in many instances to contain carbohydrate. Only by thorough treatment of the crude seromucoid fraction with absolute alcohol and ether was it possible to remove the water-insoluble constituents. The work of Kleczkowski [1938] is of interest in this connexion.

Seromucoid is similar to other proteins of the mucoid class such as ovomucoid. It is not heat-coagulable nor is it precipitated by 2 % trichloroacetic acid, but it is precipitated by alcohol and by saturated $(NH_4)_2SO_4$ and it gives a rose-red biuret and an intense Molisch reaction. The polysaccharide, accounting for 10·7 % of the molecule, contains *N*-acetylglucosamine, galactose and mannose in equimolecular proportions, and in addition there are approximately 3 % tyrosine, 1 % tryptophan and 2·3 % cystine in the preparation from ox serum. The optical rotation of $[\alpha]_{5461} - 54°$ is considerably lower than that of albumin.

The question arises, as to the relation between the "seroglycoid" of Hewitt and the apparently homogeneous fraction of the carbohydrate-containing protein incoagulable by heat, which I have here termed "seromucoid", on the ground that this name was already in use for a less purified preparation having these properties. In many respects seroglycoid and seromucoid are indeed similar. Hewitt dismissed the possibility of their identity, because his best seroglycoid preparations contained only about 10–11 % of carbohydrate, whereas Bywaters had given the carbohydrate content of seromucoid as in the neighbourhood of 20 %, and my own earlier preparations similarly contained 22 % carbohydrate [Rimington, 1933]. It will be seen, however, that when a substance with the properties of seromucoid is thoroughly purified from accompanying peptone-like substances which may contain as much as 43 % carbohydrate, the carbohydrate content falls to 10·7 %, a figure very close to that for Hewitt's best seroglycoid preparations. The relation between the two substances is considered in the following paper.

The physiological significance of the protein-bound carbohydrate in serum is not known. The French school of workers and Lustig and his associates [1931] have paid some attention to this aspect of the subject, and fluctuations in protein-sugar under certain conditions are reported. Bywaters also stated that there was less seromucoid in the serum of a starving animal than in one well-fed, but he did not carry out exact determinations.

From the immunological point of view, it is naturally of interest to know whether the polysaccharide present in the serum proteins behaves like a haptene or influences in any way the immunological specificity. Recently Coghill & Creighton [1938], using Rimington's polysaccharide prepared under such conditions that any acetyl groups would remain intact, have been unable to demonstrate such activity. Neuberger & Yuill [1940], dealing with ovalbumin and ovomucoid, have also failed to find any evidence that the carbohydrate complexes of these proteins enter into or influence in any way their immunological specificities.

Summary

Seromucoid, the carbohydrate-rich, non-coagulable protein of serum has been reinvestigated and an improved method for its isolation worked out, leading to a main fraction of uniform properties.

This contains 10·7 % of carbohydrate, consisting of *N*-acetylglucosamine, galactose and mannose in equimolecular proportions, and also approximately 3 % tyrosine, 1 % tryptophan and 2·3 % cystine. The specific rotation ($[\alpha]_{5461} - 54°$) is lower than that of serum albumin.

Methods are described for the determination of non-amino sugars and glucosamine in proteins. The ratio of hexose/glucosamine was found to be 2/1 for all fractions examined.

My sincere thanks are due to Messrs Boots Pure Drug Co. and to Mr H. Calam of their staff, who kindly undertook the preparation for me, according to instructions, of crude seromucoid from 13·5 gallons of ox blood.

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113. COMPARISON OF THE GLOBOGLYCOID, CRYSTALBUMIN, SEROGLYCOID AND SEROMUCOID FRACTIONS OF NORMAL HORSE SERUM

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(*Received 29 April 1940*)

Largely as a result of his studies of the albumin fraction of serum, Sörensen [1930] developed the view that the soluble proteins comprise reversibly dissociable component systems, from which by suitable adjustment of conditions a whole series of fractions may be obtained, varying progressively in such characteristics as solubility, carbohydrate content etc. Sörensen's investigations remain a monument of precision and detail and together with his deductions form a classic monograph in protein chemistry. It is of particular significance that though all his albumin fractions were crystalline, this afforded no guarantee of their chemical "purity", since, as he showed, their solubility was not independent of the amounts of solid phase introduced into the system. His most readily soluble albumin fraction contained as much as 50 times the quantity of carbohydrate present in the least soluble fractions, intermediate fractions possessing intermediate values. The tendency of different proteins to dissociate into their component systems was shown to vary. Serum albumin presents rather an extreme case, being very readily dissociable, whereas in egg albumin the tendency to dissociation is considerably less.

The work of Hewitt [1934; 1936; 1937, 1; 1938; 1939], in which attention was focused upon the carbohydrate contents of his serum protein fractions, led him to postulate the existence of three proteins in the albumin group, viz. globoglycoid, crystalbumin and seroglycoid. Crystalbumin contained no carbohydrate, whilst the other two, as their names imply, were relatively rich in bound carbohydrate. Hewitt's crystalbumin was obtained by several successive recrystallizations, at the isoelectric point and half-saturation with ammonium sulphate, of crude crystalline albumin from native serum. Globoglycoid is the name he gave to a protein fraction, described as a globulin, which separates when twice crystallized albumin from crude serum is brought to *p*H 7·0 and half-saturated with ammonium sulphate. It is recorded as containing about 7·5% of carbohydrate (calculated as glucosaminodihexose). Seroglycoid, the best preparation of which contained 10–11% of carbohydrate, was the protein left in the mother liquors from the albumin crystallizations and was apparently purified by removing the remainders of other proteins either by heat coagulation, or salt precipitation, or both.

If Hewitt's conclusions are correct, then the crystalline specimens of albumin which still contain carbohydrate must be regarded as admixed with either one or both of the other proteins. It appeared to us that the validity of this hypothesis might be conveniently tested by applying the anaphylactic technique in addition to chemical methods, and the investigation originally planned was broadened to include also the seromucoid fraction, a study of which, by one of us, has been presented in the preceding paper [Rimington, 1940].

Plan of the experiments

Our operations have been so planned that in one main experiment we pursued a scheme of fractionation leading to ever purer globoglycoid, with a main albumin fraction as a by-product. These operations were carried out upon the proteins from 5 l. of horse serum freed from lipins by the Hardy-Gardiner process, and have been repeated on smaller quantities of native serum. In the second main experiment untreated serum was used, and the fractionation scheme adopted achieved an ever increasing purification of the serum albumin, since, after every recrystallization, it was brought to the necessary salt and *p*H conditions to remove a further crop of "globoglycoid". It will be seen that we found the formation of globoglycoid to be an inexhaustible process. From the filtrates of the first albumin crystallization, in each case, seroglycoid fractions were prepared, as detailed below, and subsidiary experiments starting with fresh lots of native serum afforded us our seromucoid samples, the method of preparation being based upon the investigations reported in the accompanying communication.

Details concerning other methods employed may be summarized as follows. Protein concentrations: calculated from the N content (micro-Kjeldahl) and the figure of 15·6 % for the N content of serum albumin [Adair & Robinson, 1930]; checked by measurement of refractive index by Zeiss dipping refractometer. Carbohydrate (hexose) content of proteins: determined by the orcinol method [Rimington, 1940]. Optical rotatory power: measured in triple field instrument using Hg green ($\lambda = 5461$ Å.). Dialysis of protein solutions: carried out against distilled water at 0° in "Visking" (cellulose) sausage skins. Precipitation of fractions, adjustments of *p*H etc.: in every instance reagents added dropwise to the mechanically stirred solutions, final *p*H being checked electrometrically.

Details of fractionation scheme no. 1

In this instance it was desired to obtain a globoglycoid preparation in as nearly pure form as possible. The crude precipitate obtained at *p*H 7·0 and half-saturation with $(NH_4)_2SO_4$ was therefore reprecipitated under the same conditions four successive times, attention being paid each time to the material left in the mother liquors. In order to obviate any difficulties in preparation that might be caused by the presence of lipins associated with the crude albumin, the serum proteins were freed from lipins by the Hardy-Gardiner process.

5 l. of filtered normal horse serum were chilled and added in the cold room with constant agitation to 15 l. of cold alcohol. Next day the precipitate was removed on large folded filter papers, washed with cold absolute alcohol, and then transferred to 6 l. of cold anhydrous ether. Exhaustion with dry ether followed in the usual manner. To reconstitute the protein solution, the resulting powder was dissolved in 5 l. of 0·85 % NaCl and filtered from a small sediment.

The globulin fraction was precipitated by adding saturated $(NH_4)_2SO_4$ until 52 % saturation was reached. After keeping overnight in the cold room, the solution was filtered on large Büchner funnels and the precipitate washed with 52 % saturated $(NH_4)_2SO_4$. One-half of the entire crude globulin was purified by successive reprecipitations at half-saturation with $(NH_4)_2SO_4$, followed by prolonged dialysis at 0° against 0·85 % of NaCl.

To the filtrate and washings from the globulin precipitate (8·4 l.) *N* acetic acid (109·2 ml.) was added dropwise, until a faint turbidity appeared. Crystallization occurred rapidly and was brought to completion in the usual way by further addition of $(NH_4)_2SO_4$ and acetic acid. The crude albumin was collected

at the centrifuge and redissolved in distilled water to a volume of 1 l. Recrystallization was effected by $(NH_4)_2SO_4$ and *N* acetic acid as before, the crystal mass being filtered off and washed by sodium acetate—ammonium acetate-acetic acid mixture [Adair & Robinson, 1930]. The filtrate from these two operations served for the preparation of seroglycoid (see later).

Hewitt's directions for the separation of crude globoglycoid were now followed, *N* NaOH being added dropwise to the mechanically stirred albumin solution (1 l.) until *p*H 7·06 (electrometric) was reached, and then an equal volume of saturated $(NH_4)_2SO_4$ which we had also adjusted with NaOH to *p*H 7·0. No precipitate formed, so to the stirred solution acetic acid, etc. were added and the crystalline albumin recovered at *p*H 4·7. It may be noted that Hewitt [1938] found it necessary to remove the bulk of the seroglycoid before separation of globoglycoid would occur under the conditions prescribed.

Our recovered crystalline albumin was redissolved in 500 ml. water and treated as before, with the result that at *p*H 7 and half-saturation with $(NH_4)_2SO_4$, a voluminous precipitate of crude "globoglycoid" now separated (Gg 1). This was filtered off and the filtrate adjusted to *p*H 4·7 to recover the albumin it contained (A). This was twice recrystallized and then dialysed, affording the "main albumin" fraction, and it was noticed that the mother liquors from these two recrystallizations were free from heat-coagulable protein.

The crude globoglycoid Gg 1 was redissolved in 400 ml. water and again brought to *p*H 7·0 and half-saturated, affording the precipitate Gg 2 and a mother liquor from which a crystalline albumin GA 1 in the form of stout prisms (Pl. 3, fig. 1) was obtained by adjustment to *p*H 4·7. GA 1 was once recrystallized before dialysis.

The globoglycoid Gg 2 was redissolved in 300 ml. water and the process repeated yielding Gg 3 and crystalline albumin GA 2 from the mother liquor. It was noticed that the suspension of Gg 3 exhibited, on stirring, a silky sheen, suggestive of crystallization or anisotropy of flow. On repetition of the process, therefore, Gg 3 was redissolved in 300 ml. of water, adjusted accurately to *p*H 7·0 and an equal volume of neutralized $(NH_4)_2SO_4$ added drop by drop whilst vigorous mechanical stirring was maintained. The globoglycoid (Gg 4) then separated only slowly and was beautifully crystalline, in the form of needles or long slender prisms (Pl. 3, fig. 2). Gg 4 was redissolved in 200 ml. and the process repeated, the globoglycoid separating (Gg 5) and albumin from the mother liquors (GA 4) both again crystallizing in the respective forms noted above. Since the amount of material available was now becoming small, the purification process was stopped at this stage and all fractions were dialysed till salt-free and then analysed, with the results shown in Table 1.

It will be seen from Table 1 that, at each reprecipitation of the globoglycoid fraction, there was left in the mother liquor a protein, which by adjustment of the *p*H to 4·7 separated in crystalline form, indistinguishable from albumin, leaving nothing in the filtrate which could be coagulated by heat. The globoglycoid itself became crystalline after the third precipitation at *p*H 7·0, but these crystals had the form of long needles, or prisms more slender than those of albumin. There is a progressive decrease in the specific rotations of the albumin fractions recoverable from the successive globoglycoid mother liquors, and the figures for the two final materials, globoglycoid 5 and GA 4—the albumin from its filtrate, are identical within the limits of experimental error. The rotatory power of the "main" albumin, separated at the beginning of the experiment, and only twice recrystallized, is appreciably higher. With regard to carbohydrate content, it may be noted that the final globoglycoid, like the final albumin

Table 1. *Analyses of main albumin, globoglycoid (Gg) and albumin recovered from globoglycoid mother liquors (GA) (lipin-free proteins)*

Fractionation 1

Fraction	Designation	Crystalline form	$[\alpha]_{5461}$	Hexose/total N
Crude globoglycoid	Gg 1	Amorphous	—	—
Main albumin (twice recrystallized) from mother liquors of Gg 1	A	Hexagonal prisms	−74·27°	0·0075
Once reprecipitated globoglycoid	Gg 2	Amorphous	—	—
Albumin from mother liquors of Gg 2	GA 1	Hexagonal prisms	−73·33°	0·0228
Twice reprecipitated globoglycoid	Gg 3	? Microcrystalline	—	—
Albumin from mother liquors of Gg 3	GA 2	Hexagonal prisms	−73·86°	0·0082
Thrice reprecipitated globoglycoid	Gg 4	Needles or fine prisms	−73·26°	Trace
Albumin from mother liquors of Gg 4	GA 3	Hexagonal prisms	−71·24°	Nil
Four times reprecipitated globoglycoid	Gg 5	Needles or fine prisms	−68·50°	Nil
Albumin from mother liquors of Gg 5	GA 4	Hexagonal prisms	−68·61°	Nil

fraction, was carbohydrate-free. The second GA fraction showed a lower carbohydrate : N ratio than the first, and no carbohydrate could be detected in the later ones.

These considerations strongly suggest that the Gg and GA fractions obtained by this procedure are modifications of the same substance, which may be obtained in part as globoglycoid by crystallization at *p*H 7·0, the remainder then appearing as the familiar crystalline albumin on readjustment of the filtrate to *p*H 4·7. It is conceivable that an equilibrium between the two forms of the same protein is established in solution, depending upon the protein, hydrogen ion and salt concentrations.

In addition to this, however, the experimental results suggest that a progressive fractionation was taking place in the direction of albumin fractions with lower specific rotatory powers [cf. Sörensen, 1930]. The main albumin with $[\alpha]_{5461} - 74{\cdot}27°$, which had undergone a relatively limited purification, must be looked upon as intermediate with regard to this property, as the results of the second scheme of fractionation show.

Details of fractionation scheme no. 2

In this instance, 1 l. of native horse serum was employed as starting material and the crude albumin was purified by repeated treatments in half-saturated $(NH_4)_2SO_4$, first at *p*H 7·0 and then, after filtration, at *p*H 4·7. By this means, a series of globoglycoid fractions was obtained, each derived from the same albumin but in a series corresponding to progressive recrystallizations of the latter. The albumin finally obtained had thus undergone six successive crystallizations at *p*H 4·7, and five globoglycoid precipitates were obtained, of which the last three were crystalline and formed only slowly in the mechanically stirred solution.

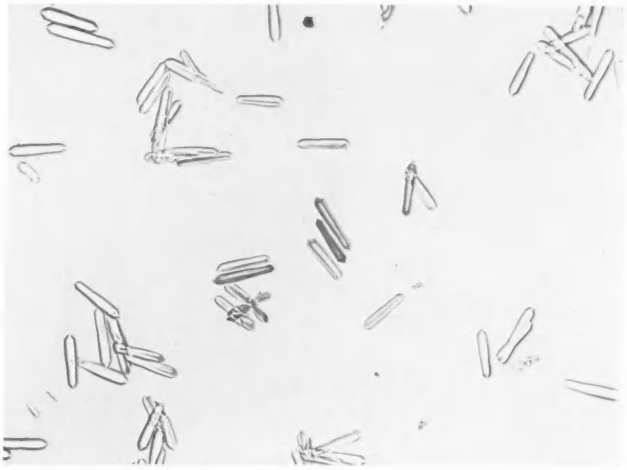

Fig. 1. Crystalbumin from horse serum. ×300.

Fig. 2. Globoglycoid from horse serum. ×220.

All solutions, when dialysed salt-free, were analysed for N and carbohydrate contents and the refractive index and specific rotatory power determined with the following results (Table 2):

Table 2. *Analysis of globoglycoid fractions and final albumin (native serum)*

Fractionation 2				
Fraction	Designation	Crystalline form	$[\alpha]_{5461}$	Hexose/total N
Globoglycoid from albumin:				
Once crystallized	CGg 1	Amorphous	$-57{\cdot}51°$	0·321
Twice crystallized	CGg 2	Amorphous	$-65{\cdot}42°$	0·160
Three times crystallized	CGg 3	Needles or slender prisms	$-70{\cdot}26°$*	0·102
Four times crystallized	CGg 4	Needles or slender prisms	$-66{\cdot}45°$	0·063
Five times crystallized	CGg 5	Needles or slender prisms	$-67{\cdot}78°$	Nil
Final albumin	CA	Stout hexagonal prisms	$-80{\cdot}79°$	0·0048

* ? High value owing to error.

It will be seen from Table 2 that this scheme of fractionation has resulted in a series of globoglycoid fractions with decreasing carbohydrate content and increasing specific rotatory power. Although CGg 1 had a hexose/N ratio of 0·321, equivalent to about 7·5% of glucosamino-dihexose, which is similar to the figure quoted by Hewitt as the carbohydrate content of his globoglycoid, the subsequent fractions contained progressively less sugar until finally in CGg 5 the quantity was too small to be determined. The final albumin, recovered after the removal of CGg 5, also contained only a trace of carbohydrate, estimated at 0·005%.

Further experiments upon the relationship between globoglycoid and albumin

Hewitt described globoglycoid as a globulin present in the albumin fraction. One would expect therefore that physical, chemical and immunological differences should exist between these two substances which would serve to differentiate them. Our experiments, on the contrary, suggest that globoglycoid is formed from crystalbumin under the conditions adopted for its isolation, or exists in equilibrium with crystalbumin in solution. Had globoglycoid been a true globulin one would have anticipated its precipitation from native serum at the first treatment with half-saturated $(NH_4)_2SO_4$. The two substances differed in their crystalline forms, and the different specimens of globoglycoid redissolved much more rapidly when placed in contact with water than did the corresponding albumin fractions, although, after stirring for some time, these also gave absolutely clear solutions. The *p*H of the redissolved and dialysed globoglycoids remained in the region of 7·0, whilst that of the albumins approximated to 5. The whole behaviour of the two substances was reminiscent of the relationship between an acid and its somewhat more soluble salt. It seems possible that some such relationship may actually exist between these two compounds. Crystalline globoglycoid was also obtained from our albumin samples when $(NH_4)OH$ was used throughout instead of NaOH for adjustment of all solutions, so that it cannot be a sodium salt. More probably globoglycoid is a combination of albumin and ammonium ions. In this connexion, one may recall the well-

known behaviour of insulin when combined with salmine whereby the region of least solubility is removed from *p*H 5·5, the isoelectric point of insulin itself, to *p*H 7·3.

McMeekin [1939] has recently reported that by acidifying a solution of carbohydrate-free crystalbumin to *p*H 4·0 with H_2SO_4 and then concentrating by ultrafiltration, he has obtained about one-half of the material in the form of crystals of an albumin sulphate; the sulphate ion was removable by electrodialysis. When boiled, dialysed solutions of globoglycoid became opalescent like milk, but, even when salts were added, gave no precipitate comparable with the coarse, curdy coagulum of heat-denatured albumin. With a view to defining more precisely the nature of globoglycoid, we undertook another fractionation experiment, in which sufficiently large fractions of the crystalline material were obtained to render possible the determination of molecular weights by osmotic pressure measurements, the reconversion of globoglycoid into crystalline albumin etc.

Fractionation 3

A quantity of once crystallized albumin (about 80 g. in all) from different batches of normal horse serum was redissolved in water, filtered from insoluble material and recrystallized at half-saturation with $(NH_4)_2SO_4$ and *p*H 4·7. This material was again dissolved, and taken through a scheme of fractionation essentially similar to that followed in fractionation 2, five lots of globoglycoid being obtained corresponding to the five successive recrystallizations of the albumin fraction.

During the course of these operations, for the purpose of following more closely the relation between globoglycoids and their parent albumins, determinations of optical rotatory power were made upon the various solutions and, since calculation of specific rotations was required, a rapid means of determining protein concentration was desirable, which should be applicable if possible to undialysed solutions.

Such a method was found in the reaction with Folin and Ciocalteu's phenol reagent as employed by Greenberg [1929]. By determining the colour value of albumin solutions of known protein content (micro-Kjeldahl and specific refractive index) the factor 22·81 was obtained for conversion of colour values with our sample of the reagent in terms of tyrosine into protein.

A high concentration of ammonium salts interfered with the phenol reagent method by causing turbidities, but since most of the solutions analysed required a 50- or 100-fold dilution to bring them into the convenient range of protein concentration, the amount of salt carried over into the final determination was too small to result in interference from this cause.

One more consideration must be mentioned. Since the factor for each protein depends upon its tyrosine or phenol content, it was possible that different factors would have to be used for albumin and globoglycoid respectively. Determinations, to be reported in a later section, showed, however, that the tyrosine and tryptophan contents of these proteins were identical, within the limits of the analytical procedure employed [Folin & Marenzi, 1929].

The phenol reagent method, although convenient, is less accurate than the Kjeldahl procedure, and it would also appear from determinations upon solutions before and after dialysis that, in the former case, the values tend to be slightly high [compare Rimington, 1924], resulting in a lower value for specific rotatory power.

The analytical results of this experiment are given in Table 3.

Table 3. *For the preparation of globoglycoid specimens. Analytical data*

Fractionation 3			
Fraction	Designation	$[\alpha]_{5461}$ before dialysis	$[\alpha]_{5461}$ after dialysis
Globoglycoid fraction from first treatment, separating on standing from mother liquor of immediate precipitate: once recrystallized	Gg 1*a*	−67·72°	—
Globoglycoid from second treatment	Gg 2	—	−68·22°
The above, twice recrystallized	Gg 2ii	−70·47°	−71·40°
Albumin recovered from mother liquors of these recrystallizations	Gg (2–2ii) alb.	−68·42°	—
Extra globoglycoid from second treatment separating after adjustment of *p*H from 6·6 to 7·0: once recrystallized	Gg 2*a*i	—	−72·38°
Globoglycoid from third treatment	Gg 3	−69·80°	−72·47°
Main albumin filtrate after removal of above	A (3)	−71·45°	—
Globoglycoid from fourth treatment	Gg 4	−73·26°	—
Globoglycoid from fifth treatment	Gg 5	−74·54°	—
Main albumin after removal of above and two recrystallizations	Final alb.	−74·11°	−74·64°

*Recrystallization of globoglycoid at p*H *4·7*

This was accomplished using a portion of the specimen Gg 3, the specific rotatory power of which had been determined both before and after dialysis, affording a mean value of −71·14°. To the protein solution was added an equal volume of saturated $(NH_4)_2SO_4$ (unneutralized) and the *p*H adjusted cautiously to 4·7 by the dropwise addition of 2·5 % acetic acid to the mechanically stirred solution. The separation of crystals commenced after the addition of 1 ml. of acid and was complete after 3·5 ml. The crystals were stout hexagonal prisms indistinguishable from crystalbumin, and the specific rotatory power determined upon a 3 % solution was −70·89°. Thus, as in the examples noted in fractionation 1, and also in the case of the final globoglycoid and albumin fractions (Gg 5 and Final alb.) of fractionation 3, there was no difference between the specific rotatory powers of the globoglycoid and the crystalline product derived from it or from its mother liquors at *p*H 4·7. These two materials, obtained at *p*H 7·0 and at 4·7 respectively, did however exhibit the customary difference, the former being in the form of long, slender prisms or needles, and the latter being indistinguishable from ordinary crystalline albumin.

A determination of the osmotic pressure of globoglycoid at *p*H 7·0 was kindly carried out for us by Dr Elford and afforded values corresponding to a mol. wt. for the protein of approximately 60,000 uncorrected for ionic effects.

Preparation of seroglycoid and carbohydrate-rich albumin fractions

From a study of Hewitt's papers dealing with the protein fraction he has named seroglycoid, it is clear that several alternative routes are available for arriving at a concentration of the carbohydrate-rich material left in the filtrate

after the separation of crystalline albumin from serum. Thus the second communication [Hewitt, 1937, 1] describes under "Fractionation methods" a separation by means of 2/3 saturated $(NH_4)_2SO_4$, and trichloroacetic acid is there stated to give inconsistent results. Later much attention is devoted to the production of fractions rich in carbohydrate by a process involving treatment in the boiling water bath at two different hydrogen ion concentrations. In a subsequent paper [Hewitt, 1937, 2] dealing with sera from various animal species, salting out, trichloroacetic acid precipitation, heat coagulation and combinations of these processes are all employed. For comparison with our own fractions we decided to make material by both the heat coagulation technique and by a scheme of fractionation at the 66, 75 and 100% levels of saturation with $(NH_4)_2SO_4$ [see Lustig, 1930]. The latter preparations were used in the anaphylaxis experiments since the application of heat at any stage is undesirable when the preparations are intended for immunological work. Trichloroacetic acid, in our hands, proved to be unsatisfactory. Our preparations, though we make no claim for their homogeneity, should, at least, have contained appreciable quantities of seroglycoid.

The filtrate from fractionation 1, after the albumin had been removed as completely as possible by crystallization at *p*H 4·7, was brought to *p*H 6·0 by NaOH and then mechanically stirred in a large flask immersed in a water bath. The solution was maintained at 80° for 15 min., then brought to *p*H 4·7 and again heated for 15 min. Next day the coagulum was filtered off and the filtrate (11 litres), after being dialysed, was concentrated under reduced pressure to 300 ml. 2 vol. of alcohol were added and the precipitate produced was centrifuged off, and redissolved in water. The solution was filtered from some inorganic matter and then reprecipitated with alcohol. After a further reprecipitation the material was dissolved, dialysed and stored after filtration through a Seitz filter. This material gave an intense Molisch reaction and a purplish-pink biuret reaction.

Another preparation was made from native serum in an analogous manner, but, in order to avoid the lengthy process of concentration under reduced pressure, the filtrate from the albumin was fully saturated with $(NH_4)_2SO_4$ and the precipitate thus produced was redissolved and heated at 80° as described above. Trouble was encountered, when attempting to redissolve the alcohol-precipitated material, from the presence of insoluble, lipoid material giving strongly positive sterol reactions [compare Rimington, 1940].

Ammonium sulphate fractionation was carried out on the albumin filtrate derived from 2 l. of serum. By raising the concentration of this salt to 66% saturation a fairly bulky precipitate (Sg 66) resulted which, when redissolved and dialysed, gave a deep brown solution. Further raising of the concentration in the main filtrate to 75% saturation afforded Sg 75, redissolving to a straw-coloured solution, and finally by full saturation there resulted a scanty precipitate (Sg 100) giving a slightly opalescent but almost colourless solution. These fractions were dialysed and their hexose : N ratios determined. For comparison, the analytical results on preparations from two additional sera are also set out (Table 4).

Table 4. *Ratio* $\frac{\text{Hexose}}{\text{total N}}$ *for ammonium sulphate fractions*

	Sg 66	Sg 75	Sg 100
Serum 1	0·313	—	—
Serum 2	0·453*	0·548	1·370
Serum 3	0·315	0·538	1·370

* Reprecipitated at $\frac{2}{3}$ saturation.

Preparation of seromucoid fraction

Since the proper purification of seromucoid demands much labour and considerable quantities of starting material, preparations were employed which were obtained as follows: 5 l. serum were diluted with 15 l. of water and adjusted to *p*H 4·7 with acetic acid. The mixture was then heated in a steam-jacketed pan until coagulation was complete and the *p*H readjusted, if necessary, to 4·7. The coagulum was filtered off and the clear, yellow filtrate concentrated under reduced pressure to a small volume and then precipitated by 2 vol. of alcohol. The precipitate was centrifuged down and washed several times with absolute alcohol, which removes, among other things, much pigment, and then with ether. After resuspension in water, a small insoluble residue was removed, affording a water-clear solution giving an intense Molisch reaction. Analyses indicated a hexose : N ratio of 0·574, corresponding approximately to 11·2 % of glucosaminodihexose, since the N content of seromucoid is only about 13 %.

Determination of tyrosine and tryptophan contents of the various serum protein fractions

A further basis for the comparison of the different fractions was afforded by their tyrosine and tryptophan contents. The Folin & Marenzi [1929] method was used and the following results obtained:

Fraction	Tyrosine	Tryptophan	Hexose/total N
Main albumin (A) from fractionation 1	4·17	0·29	0·0075
Globoglycoid (Gg 5) five times reprecipitated, according to fractionation 1	4·29	0·30	Nil
Precipitate (Sg 66) formed by $\frac{2}{3}$ sat., with $(NH_4)_2SO_4$ after removal of crystalline albumin	4·59	1·04	0·272
Precipitate (Sg 75) formed by $\frac{2}{3}$ sat. after removal of Sg 66	4·05	0·99	0·313

These figures for tryptophan content confirm those of Hewitt, who found 0·26–0·30 % in his rigorously recrystallized albumin samples of low carbohydrate content and values around 1·0 % for the carbohydrate-rich seroglycoid fractions. His tyrosine values are somewhat higher than ours, but this may perhaps depend upon the method of calculation, since the N content of the carbohydrate-rich fractions is appreciably lower than that of the recrystallized albumins. Hewitt, however, used the factor $N \times 7{\cdot}0$ for calculation of the protein content of his materials.

IMMUNOLOGICAL INVESTIGATIONS

In the immunological comparison of several protein fractions from one serum, many difficulties arise, which are mainly due to the presence in any one fraction of traces of one or more of the others.

The preparation of antisera, and their absorption to render them specific, are laborious procedures. Nevertheless Hewitt [1937, 3] has succeeded by such methods in demonstrating immunological differences between seroglycoid and crystalbumin from horse serum. He concluded moreover that pure, carbohydrate-free crystalbumin was but poorly antigenic in comparison with seroglycoid or globulin. He attributed the main antigenic function of his crystalbumin

preparations to the presence of traces of seroglycoid and globulin. Dale & Hartley [1916] had previously shown that the sensitivity to the albumin fraction of horse serum requires a longer latent period for its development than sensitivity to horse serum globulins. If a mixture of antigens consisting of albumin, globulin and seroglycoid is used for immunization, it is to be expected that precipitins for the better antigens (e.g. globulin) will develop more rapidly than precipitins for albumin.

The test of the anaphylactic responses of isolated uterine strips from actively sensitized guinea-pigs affords a rapid, simple, and highly specific method for the comparison of the antigenic properties of serum fractions. The method has been successfully employed by Dale & Hartley in a study of the immunological differences between the globulins and albumins of normal horse serum. Hewitt did not succeed in demonstrating immunological differences between his serum fractions by anaphylactic methods. His experiments, however, were performed on the whole animal, and massive, shock-eliciting doses of the antigens were injected. A method using the isolated tissue is better suited to this type of experiment, since desensitization of the whole guinea-pig to several antigens is impracticable, whereas a single sensitized smooth muscle strip suspended *in vitro* can be used for several successive tests with different antigens.

In the majority of our experiments specific sensitiveness was induced by a single intraperitoneal injection, of 2 mg. of a separated protein fraction, into a normal virgin guinea-pig weighing 125–150 g. A latent interval of at least 3 weeks was allowed after the sensitizing injection. In agreement with the findings of Dale & Hartley, an interval of this duration was found to be essential for the development of adequate sensitiveness to our crystalbumin preparations. We have, indeed, in this way repeatedly succeeded in inducing specific sensitivity to crystalbumin, without at the same time producing sensitivity to globulin, and in some experiments with a preparation of very highly purified ten times recrystallized crystalbumin.

In a few cases guinea-pigs were passively sensitized by a single I.P. injection of 0·5 ml. of an antiserum from a rabbit immunized to a single protein fraction.

Groups of guinea-pigs were sensitized to the most highly purified samples of each of the five serum fractions to be compared. These were: (1) the six-times precipitated total globulins from horse serum; (2) the crystalbumin from lipin-free serum after it had been crystallized repeatedly and freed before each crystallization from "globoglycoid" (CA); (3) globoglycoid which had been repeatedly reprecipitated at *p*H 7·0 in 50% sat. $(NH_4)_2SO_4$ (Gg 5); (4) seroglycoid, represented by that fraction of normal horse serum which after complete removal of crystalbumin is precipitated at $(NH_4)_2SO_4$ concentrations between 66 and 75% saturation (Sg); (5) seromucoid (Sm).

For the comparison of any two of these fractions, the excised uterine strips, from a guinea-pig sensitized with one of them, were suspended in Locke's solution, according to Dale's [1913] method. One of the uterine strips was then tested for specific sensitiveness to the homologous (sensitizing) antigen, and the other for its ability to react to the other serum fraction under test.

With the possible exception of crystalbumin and globoglycoid, the serum fractions used in these investigations were not immunologically pure. In the comparison of any two serum fractions, therefore, the presence of traces of a third fraction as a common impurity could yield results which might erroneously suggest the existence of some degree of antigenic community between the two fractions. In a large number of the earlier experiments, much confusion resulted from the presence of such impurities. It became necessary therefore to

adopt a technique whereby the sensitiveness to those antigenic fractions which, for any particular experiment, could be regarded as impurities, was specifically neutralized, before the two antigens to be compared were tested. Weil [1914] has shown that specific desensitization is only relative. Sensitized smooth muscle, after reacting specifically with the sensitizing antigen in high dilution, is no longer capable of reacting to the antigen in that dilution, but still reacts to the same antigen in a higher concentration. In the majority of our experiments the test doses were applied at a constant dilution. The plain muscle sensitized with antigen A was first tested with antigen B, freedom of which from A could not be guaranteed. It might give a reaction to the first application of B and thereafter show itself insensitive to a further similar dose of B, but still react to the same dose of the specific antigen A. Such a result of the incomplete purity of several of the antigens must be kept in view in interpreting the results. The method evolved may be illustrated by the results of actual experiments. The interval allowed for sensitization after injection of the antigen is to be understood to have been 3 weeks, unless otherwise indicated.

(1) *Plain muscle from guinea-pigs sensitized to crystalbumin (CA) or to globoglycoid (Gg)*

The earlier experiments of this group were directed to a comparison between crystalbumin and globoglycoid, which, according to the chemical evidence already presented, seemed to be interconvertible forms of the same protein. The initial responses given by the plain muscle to either of these proteins were, in fact, indistinguishable, whichever had been used for the sensitizing injection. When, however, attempts were made to desensitize the plain muscle to either Gg or CA by increasing and repeated applications of one of them, a phenomenon suggestive of some kind of antigenic disparity between the two presented itself. If, after desensitization to a relatively high concentration of one, the uterus was then tested with the other protein in low concentration, in a number of experiments it gave a pronounced reaction. Fig. 3 illustrates this phenomenon. It shows that the application of 1 mg. of either Gg or CA to the plain muscle, no longer capable of responding to 0·2 mg., resulted in a typical contraction, after which it was insensitive to further application of the larger dose. Each was still however capable of reacting to the other in the original lower concentration. We have records as clear as that shown in Fig. 3, from plain muscle of guinea-pigs sensitized with CA, giving large responses to 0·2 mg. of Gg or of CA, after complete desensitization to 1 mg. of the other. In other cases the phenomenon was less clear. For example, plain muscle from a guinea-pig sensitized with Gg, after being completely desensitized to 2 mg. of CA may give only a small residual reaction with even 2 mg. of Gg.

The explanation of this phenomenon is not easy. A supposition that CA and Gg are essentially the same protein with a common antigenic specificity, as the chemical evidence would lead one to expect, but that our preparations of them are incompletely pure, and that the contaminant is a different additional antigen in each case, is incompatible with the reaction observed; for application of the antigen which had been used for sensitization should remove also the sensitiveness to the other. Such a suggestion is moreover difficult to reconcile with the fact that the phenomenon under discussion is exhibited as clearly and strongly by plain muscle from guinea-pigs sensitized with our purest crystalbumin, which gives no reaction with globulin, as by that from guinea-pigs sensitized with less rigidly purified samples of CA. It would be possible on the other hand

to suppose that CA and Gg are separate sensitizing antigens and that our preparation of each of them contains the other as contaminant. Numerous experiments, however, and especially those in which plain muscle from guinea-pigs

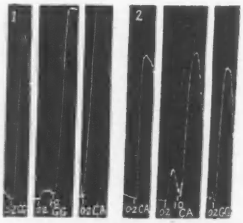

Fig. 3.

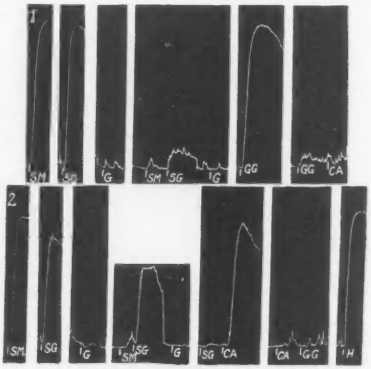

Fig. 4.

Fig. 3. Response *in vitro* of the uterine muscle of a guinea-pig sensitized with globoglycoid. 1. First horn. 0·2 Gg, 0·2 mg. globoglycoid; CA, crystalbumin. 2. Second horn.

Fig. 4. Response *in vitro* of the uterine muscle of a guinea-pig sensitized with crystalbumin. 1. First horn. Sm, seromucoid; Sg, seroglycoid; G, globulin; Gg, globoglycoid; CA, crystalbumin. 2. Second horn. H, 0·002 mg. histamine.

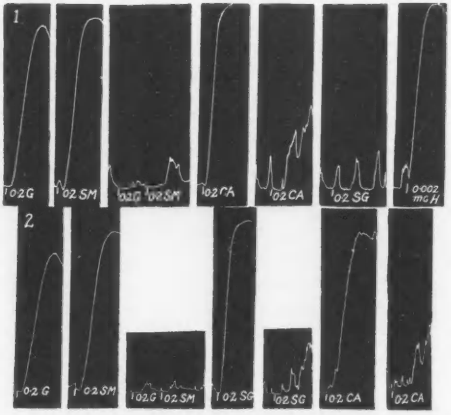

Fig. 5. Response *in vitro* of the uterine muscle of a guinea-pig sensitized with crystalbumin. 1. First horn. 0·2 G, 0·2 mg. globulin; Sm, seromucoid; CA, crystalbumin; Sg, seroglycoid; H, histamine. 2. Second horn.

passively sensitized with rabbit antigloboglycoid or anticrystalbumin serum were used, and which will be described subsequently, have shown that Gg and CA cannot be regarded as separate antigens.

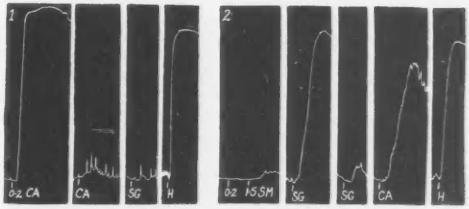

Fig. 6. Response *in vitro* of the uterine muscle of a guinea-pig passively sensitized with rabbit anti-albumin serum. 1. First horn. 2. Second horn. The test dose of protein was 0·2 mg. throughout, except in the case of the second application of seromucoid (Sm) to the second horn, when the dose was 1·5 mg. CA, crystalbumin; SG, seroglycoid; H, 0·002 mg. histamine.

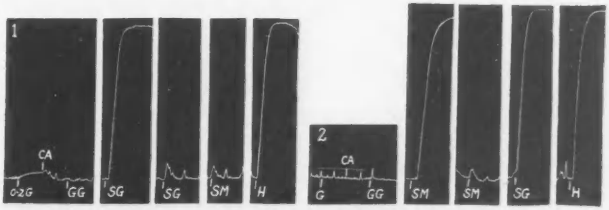

Fig. 7. Response *in vitro* of the uterine muscle of a guinea-pig sensitized with seroglycoid. Test dose of 0·2 mg. protein used throughout. 1. First horn. 0·2 G, 0·2 mg. globulin; CA, crystalbumin; Gg, globoglycoid; Sg, seroglycoid; Sm, seromucoid; H, 0·002 mg. histamine. 2. Second horn.

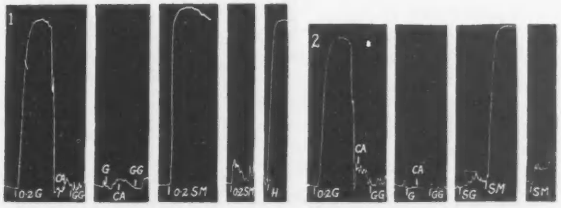

Fig. 8. Response *in vitro* of the uterine muscle of a guinea-pig sensitized with seromucoid. Test dose of 0·2 mg. protein used throughout. 1. First horn. 0·2 G, 0·2 mg. globulin; CA, crystalbumin; Gg, globoglycoid; Sm, seromucoid; H, 0·002 mg. histamine. 2. Second horn. Sg, seroglycoid.

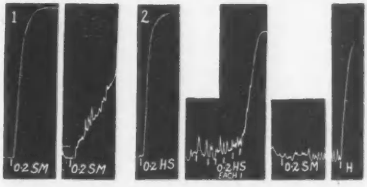

Fig. 9. Response *in vitro* of the uterine muscle of a guinea-pig sensitized with seromucoid. 1. First horn. 0·2 Sm, 0·2 mg. seromucoid. 2. Second horn. 0·2 Hs, 0·2 ml. normal horse serum; H, 0·002 mg. histamine.

Another possible explanation seems to be that CA and Gg, which the chemical evidence represents as different but interconvertible physical forms of the same protein, act as a single sensitizing antigen, so that either form produces the same degree of primary sensitiveness to both, but that the physical differences confer a minor degree of antigenic disparity, such that desensitization to CA will leave sensitiveness to Gg and *vice versa*.

In subsequent experiments we tested the reactions of plain muscle from guinea-pigs, actively sensitized with CA or Gg, first to seromucoid, seroglycoid and globulin, and its reaction to the specific sensitizing antigen, after desensitization to these others. Fig. 4 shows the result of such an experiment. There was a full reaction to Sm, and subsequent application of Sg also evoked a clear and characteristic but slightly smaller reaction. After these responses there was no reaction to globulin (G), so that, if G contained any of the antigens responsible for the responses to Sm and Sg, it contained not more, and probably less, than Sm or Sg. Further applications of these three non-specific antigens completed the desensitization to all, which was less easily effected to Sg than to the others. The plain muscle was then treated with Gg and CA in the standard doses. It still reacted well to either, although the reaction was not in either case as complete as would be expected if the muscle had not been partially desensitized by the earlier treatment with the other antigens; but it was at least as strong with Gg as with CA, the antigen with which the animal was sensitized. The plain muscle was then no longer capable of responding to either CA or Gg. On this evidence it would be reasonable to conclude that CA and Gg, if both could be obtained free from traces of other proteins, will be antigenically indistinguishable.

In the experiment illustrated in Fig. 5 both horns of a uterus actively sensitized to CA were first desensitized to G and Sm. One of the horns was then still capable of responding once with maximal reaction to CA but not to subsequent contact with CA or Sg. The other horn gave a typical reaction to Sg, after which it was no longer able to respond to the same dose of Sg, but application of CA was followed by a definite but submaximal reaction. Fig. 5 can be explained by the supposition that our G, Sm and Sg preparations all contain antigenic constituents in common with the sensitizing antigen CA. The fact that the response to our Sg significantly reduces the sensitivity and subsequent response to CA is probably explicable by the presence in the Sg of a significant contamination with CA which the method used for purifying Sg would not be likely to exclude.

A number of experiments was performed with the plain muscle from guinea-pigs passively sensitized with antiserum from rabbits which had received two courses of immunizing injections of our purest crystalbumin preparation. (The total amount of crystalbumin received by each rabbit during the two courses was 24 mg.) Such antisera could reasonably be expected to contain antibodies, the bulk of which are specific for the main antigenic constituent of the preparation used for immunization (CA). The sensitivity induced by such an antiserum will therefore be predominantly for CA, and sensitivity to the minor antigenic components (impurities) of our crystalbumin preparation will be less striking than after active sensitization with CA, when the respective degrees of sensitivity induced to the minor components of the antigen may be important and even as great as that to the main component. Fig. 6 shows the result of an experiment in which the uterine muscle of a guinea-pig sensitized by the intraperitoneal injection of 1·5 ml. of anticrystalbumin serum was used, and from it can be concluded that Sm contained no appreciable amount of crystalbumin but that our Sg contained a significant CA component.

The plain muscle from guinea-pigs passively sensitized with anticrystalbumin or antigloboglycoid serum was found, moreover, to react equally to both Gg or CA, and application of either of these preparations resulted in desensitization to both. The responses to G, Sg and Sm of plain muscle sensitized to CA or Gg are not unexpected in view of the fact that none of the other fractions could be regarded as purified to the same extent as the crystallizable fractions (CA and Gg). It is indeed evident that our Sg, for example, contained appreciable amounts of crystalbumin; the seromucoid preparation, on the other hand, was apparently free from CA. The majority of results have shown, in addition, that crystalbumin and globoglycoid are antigenically indistinguishable; and only in a number of the earlier experiments have minor immunological differences, the nature of which is at present obscure, been observed between them.

(2) *Plain muscle from guinea-pigs sensitized to seroglycoid (Sg) or to seromucoid (Sm)*

The responses of uterine plain muscle of guinea-pigs actively sensitized by a single injection of 2 mg. seroglycoid have been variable. In the majority of cases, typical anaphylactic responses have been obtained by contact with any one of five serum fractions available. This finding was not unexpected in the light of evidence which has already been provided that our Sg, owing to its method of preparation, could not be regarded as immunologically pure. It has, however, occasionally been found that sensitiveness to Sg could be induced without at the same time producing demonstrable sensitiveness to CA, Gg or globulin. The result of such an experiment is shown in Fig. 7. From this result it must be concluded that seroglycoid contains an antigen, immunologically distinct from G, CA or Gg. This antigen is, moreover, present also in our Sm preparations, although in significantly smaller amounts than in Sg.

The antigenic difference between Sm and Sg could be demonstrated even more strikingly by the use of plain muscle from guinea-pigs actively sensitized to Sm. Fig. 8 shows the result of a typical experiment in which the uterine plain muscle after desensitization to the standard dose of 0·2 mg. of G, CA and Gg, was incapable of reacting to Sg, and application of Sg did not appreciably reduce the sensitivity to subsequent contact with Sm. Sm, therefore, owes its principal antigenic properties to a component different from the main antigen of Sg.

A few experiments were performed with the plain muscle of guinea-pigs passively sensitized by single intraperitoneal injections of an antiserum from rabbits immunized against our seroglycoid preparation. Such plain muscle was found capable of responding in a typical manner to contact with CA, but after desensitization to this antigen it was still capable of reacting to contact with either Sm or Sg. A single response to Sg was, however, followed by desensitization to both Sm and Sg, whereas the response to Sm resulted in desensitization to this antigen only, leaving the plain muscle capable of reacting to subsequent contact with Sg. The conclusions which can be drawn from these results with passively sensitized plain muscle are identical with those to be drawn from the experiments with actively sensitized material, namely that our Sg and Sm are immunologically different although our preparations of them contain a common antigen. This might be a common impurity, but it seems more probable that our Sg contained traces of Sm.

The observed immunological differences between our Sg and Sm preparations were apparently not brought about by the drastic methods of extraction and

purification involved in the preparation of the Sm. This was proved by the demonstration that sensitivity induced by Sm could be completely neutralized by the application of untreated horse serum (see Fig. 9).

Discussion

We have prepared those fractions which Hewitt has designated "globoglycoid", "crystalbumin" and "seroglycoid" by following as closely as possible the methods he has described. We have also prepared seromucoid for comparison. By applying to normal serum different schemes of fractionation, we have in the one case proceeded to ever purer crystalbumin, by eliminating globoglycoid before each of the six successive crystallizations. In the other case, by subjecting the globoglycoid formed at the first treatment to repetition (three successive times) of the process of precipitation with half-saturated $(NH_4)_2SO_4$ at the neutral point, a purification of the globoglycoid was achieved, so that it became crystalline, and it was possible to remove albumin in crystalline condition from the mother liquors after each precipitation. The albumin from which the first crude globoglycoid had been separated was once recrystallized and gave us our main albumin fraction.

Hewitt has claimed that globoglycoid behaves like a globulin, is amorphous and contains carbohydrate, but we have repeatedly succeeded in obtaining it in a crystalline condition. The carbohydrate associated with the crude material was found to decrease with each successive reprecipitation or recrystallization, until finally a product was obtained which was carbohydrate-free, and chemically indistinguishable from crystalbumin (amino-acid content, mol. wt. etc.) and different only in certain physical properties, such as crystalline form, and appearance after heat coagulation, which may depend upon the state of dissociation and combination with anions or cations, as regulated by the *p*H of the medium with which the protein is in contact.

Particular interest attaches to the determination of optical rotatory power made on the materials resulting from the two main schemes of fractionation. It would appear that the crude globoglycoid separating from albumin upon the initial treatment at *p*H 7·0 possesses a relatively high optical rotatory power, but that as this globoglycoid is reprecipitated or recrystallized under the same conditions its optical rotatory power diminishes. The albumin recoverable at *p*H 4·7 from the final globoglycoid mother liquor has the same low value as the globoglycoid itself. When, on the other hand, successive crops of globoglycoid are taken off from the same initial albumin, these show a progressively increasing rotatory power, until finally a globoglycoid is obtained, having a moderately high optical rotatory power, identical with that of the albumin in its mother liquor. These observations can be most readily explained on the hypothesis of mutually interacting component systems as postulated by Sörensen [1930]. As has been pointed out in the experimental section of this paper the carbohydrate which is associated with the crude globoglycoid fraction is lost upon reprecipitation and recrystallization of the latter, just as is that from crude crystalbumin, and both these materials may ultimately be obtained practically carbohydrate-free.

On the immunological side, obvious difficulties of interpretation arise when anaphylactic sensitization and the subsequent testing of the specific sensitivity are carried out with proteins which cannot be regarded as rigidly purified from others having different antigenic specificities. For example, if plain muscle from an animal sensitized to crystalbumin is found to exhibit a minor degree of sensitiveness to globulin, and still to react to the albumin when desensitized to

a larger dose of the globulin, it is obvious that the condition might be attributable either to a trace of globulin in the albumin used for the sensitizing injection, or to the presence of albumin as a minor constituent of the globulin used for testing. The antigenic distinctness of globulin and albumin has been sufficiently established by previous work, and the method used by us for purifying our globulin fraction offers no good guarantee of its purification from the other serum antigens. It seems proper, therefore, to attach little weight, in our interpretations, to the reactions to globulin shown by plain muscle from guinea-pigs sensitized to the other proteins studied. Eliminating these, we are left with evidence of an almost complete community of antigenic specificity between globoglycoid and crystalbumin, corresponding to the chemical evidence that they represent essentially the same protein, probably in different combinations with inorganic ions producing two different points of minimum solubility.

Our seroglycoid preparations were certainly impure, and the method of preparation could hardly be expected to lead to the isolation of a single protein from so complex a mixture, but determinations of carbohydrate content indicated that in these fractions there accumulated a protein, the chemical properties of which are markedly different from those of crystalbumin. It is relatively rich in bound carbohydrate (hexose/N ratio approximately 0·3) and contains more than twice as much tryptophan and slightly more tyrosine than does crystalbumin. The seromucoid preparations were also carbohydrate-rich (hexose/N ratio 0·57). Immunologically, also, there was evidence that our seroglycoid and seromucoid were impure, but that each contains an antigen distinct from any present in the other proteins studied, presumably due to their characteristic glycoproteins, and antigenically distinct with respect to one another. Our results, however, give no indication as to whether the specificity is due to the association of two different protein antigens with a common antigenically indifferent carbohydrate complex (cf. Coghill & Creighton [1938]; Neuberger & Yuill [1940]), or to two distinct carbohydrate haptenes combined with the same protein.

Combining our conclusions with those from earlier work, it appears that the test based on anaphylaxis will detect the presence in normal serum of at least five protein antigens—euglobulin, pseudoglobulin, crystalbumin (existing also in the form of globoglycoid), a glycoprotein in "seroglycoid" and another in "seromucoid". Our conclusions agree with those of Hewitt in regarding the carbohydrate complex found in crude crystalline serum albumin as associated with other protein constituents than the crystalbumin itself, which when rigidly purified contains no carbohydrate. They differ, in that we find that the fraction which Hewitt termed "globoglycoid", regarding it as a globulin associated with carbohydrate, is not a globulin in any sense except its precipitation by $(NH_4)_2SO_4$ at neutral reaction; on the contrary we find it to be completely crystallizable, interconvertible with crystalbumin by appropriate methods, and similarly free from carbohydrate when pure.

Summary

Methods of preparation of crystalbumin, globoglycoid, seroglycoid and seromucoid from horse serum are described.

Chemical and immunological investigations (anaphylaxis *in vitro*) on the separated fractions have been undertaken. From the results of these it is concluded that crystalbumin and globoglycoid are identical or closely similar. Seroglycoid is found to differ quantitatively from crystalbumin in its carbohydrate and amino-acid contents.

Immunological evidence is provided which suggests that seroglycoid preparations contain a carbohydrate antigen which is distinct from crystalbumin.

Seroglycoid and seromucoid, although both rich in bound carbohydrate, differ from one another when compared by anaphylactic methods.

We wish to express our thanks to Mrs M. E. Adair for kindly supplying several protein solutions which we used for comparison with our own preparations, and for her interest in this investigation.

We are indebted to Sir Henry Dale for his invaluable and helpful criticism.

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114. THE IRON OF THE PLASMA

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(*Received 10 April 1940*)

The iron content of the plasma is small but it is probably of great importance as transport iron. In the normal subject the range is narrow and results obtained by various workers within recent years have shown general agreement, the figures ranging from about 0·1 to 0·2 mg. Fe per 100 ml. [Barkan, 1927; Fowweather, 1934; Tompsett, 1934; Moore, 1937].

Barkan [1925] found that plasma Fe was not ultrafiltrable but became so after incubation of the plasma with dilute HCl at 37°. Tompsett [1934] found that only a small fraction of the plasma Fe could be recovered in trichloroacetic acid extracts and that added Fe (as iron alum) could not be recovered quantitatively in such extracts. Fowweather [1934] made a similar observation. The writer has found that plasma Fe and added Fe could be recovered quantitatively in trichloroacetic acid extracts if thiolacetic acid, a reducing agent, had previously been added. It has also been found that whereas ferrous Fe could be recovered quantitatively in trichloroacetic acid extracts of materials containing phosphatides or phosphoproteins, ferric Fe could not. Plasma Fe was shown to be in the ferric state *in vitro*. In later work, Barkan [1937] noted that the plasma Fe could be recovered quantitatively in trichloroacetic acid extracts provided that the plasma had previously been incubated with dilute HCl at 37° for 24 hr.

The following experiments were carried out in order to obtain a clearer insight into the nature of the plasma Fe and the Barkan phenomenon.

In the first experiments, the influence of plasma on the dialysis of ferrous and ferric salts was studied. To 5 ml. of plasma was added 1 mg. Fe (ferrous sulphate, iron alum, iron ammonium citrate) and the mixtures were dialysed against 15 ml. water for 4 hr. At the end of this period the Fe contents of the dialysates were estimated with thiolacetic acid and NH_3 [Tompsett, 1934]. Typical results are shown in Table 1, where it will be seen that whereas ferrous Fe dialysed easily, ferric Fe dialysed hardly at all.

Table 1. *Typical results obtained in a study of the dialysis of added Fe from plasma*

1 mg. Fe added; the figures represent mg. Fe that have dialysed in 4 hr.

Ferrous sulphate	0·30
Iron alum	0·01
Iron ammonium citrate	0·01
Iron alum (plasma containing 0·8% HCl)	0·18

Tompsett [1940] has shown that certain foodstuffs are capable of reducing ferric Fe to the ferrous state, particularly at acid reactions. It was suggested that proteins were chiefly responsible for this phenomenon. It seemed possible that serum and plasma could have this property. 1 mg. Fe (as iron alum) was added to 5 ml. plasma. On standing no appreciable reduction occurred. On standing at acid reactions reduction did occur, as evidenced by the fact that

after standing 1 hr. in the presence of 0·8 % HCl, a red colour developed on the addition of αα′-dipyridyl.

The dialysis of ferric Fe from acidified plasma (0·8 % HCl) was then examined. As will be seen from the results shown in Table 1 ferric Fe appeared to dialyse readily under these conditions. Upon examination with αα′-dipyridyl, it was found that the whole of the Fe in the dialysates was in the ferrous state.

The question naturally arises as to the nature of the substances producing this reduction. It might be due to ascorbic acid; the writer, however, is rather of the opinion that the plasma proteins are responsible. Ascorbic acid has been found to reduce ferric Fe as readily in neutral as in acid solution, whereas in the case of plasma an acid reaction appears to be necessary or to be very definitely favourable. This problem requires further investigation.

Discussion

The Fe present in samples of plasma obtained under conditions which do not prevent the re-oxygenation of haemoglobin is in the ferric state. This accounts for the fact that the plasma Fe, unless subjected to special treatment, is not ultrafiltrable. The phosphatides are probably more concerned with this phenomenon than the proteins.

It is now possible to explain the "Barkan phenomenon". It has been shown that the plasma Fe becomes reduced to the ferrous state when the plasma is acidified and allowed to stand. Ferrous Fe is ultrafiltrable.

Barkan has referred to the plasma Fe as the "easily split iron". This term would suggest that such Fe is present as a compound distinct from complexes of Fe which are generally recognized. The Fe of the plasma is probably present as a salt, although undissociated, with such substances as phosphatides, proteins etc. There is no reason to suggest that it is combined with any particular substance or group of substances and it is quite likely distributed amongst a number of them. In view of the facts discussed above, the writer is of the opinion that the term plasma Fe is a sufficient description.

The Fe of the plasma has been found to be wholly in the ferric state and as such undissociated. This does not indicate that this is the permanent state of the plasma Fe *in vivo*. It seems very probable that at some stage the plasma Fe will, at least in part, be changed into the ferrous state, i.e. become capable of dissociation and ultrafiltrable. That this must occur seems obvious, for otherwise it seems difficult to visualize how the plasma Fe, if it is to be regarded as transport Fe, is to become utilizable.

Summary

The "Barkan effect" observed in plasma and serum has been shown to be due to the reduction of the plasma Fe to the ferrous state with a consequent change from a non-ultrafiltrable to an ultrafiltrable condition.

I wish to thank Dr A. B. Anderson for his helpful criticism and advice.

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115. FACTORS INFLUENCING THE ABSORPTION OF IRON AND COPPER FROM THE ALIMENTARY TRACT

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(*Received 10 April 1940*)

Iron

Although the importance of Fe in respiratory processes has been recognized for a long period, there are still gaps in our knowledge of the many processes in which this element is involved, e.g. very little is known concerning the absorption of Fe from the alimentary tract. The work to be described is concerned with this problem.

The Wisconsin workers [Hart *et al.* 1928; Elvehjem, 1932] have produced evidence to show that "inorganic" Fe but not haematin Fe can be absorbed from the alimentary tract and subsequently converted into haemoglobin.

There has been much controversy as to whether both ferrous and ferric Fe or only the ferrous form can be absorbed as such. Heubner [1926] and Lintzel [1931] state that ferric but not ferrous Fe forms non-dialysable compounds with proteins and they believe that ferric Fe cannot be absorbed as such but must first be reduced to the ferrous state. They consider the intestine to be the site of this reduction as it contains easily oxidizable materials and has a low oxygen tension. This view is supported by Starkenstein & Weden [1930], Reimann & Fritsch [1930] and Moore *et al.* [1939]. Other workers believe that ferric Fe can be absorbed as such [Whipple & Robscheit-Robbins, 1936; McCance & Widdowson, 1937; 1938; Brock & Hunter, 1937]. These workers base their views on the following considerations: (1) in balance experiments, ferric Fe has been found to be absorbed as easily as ferrous Fe; (2) in the treatment of anaemia, ferric Fe often produces as good a response as ferrous Fe. Those who hold the first view have in no case produced any evidence that reduction of ferric Fe does take place in any part of the alimentary tract.

Tompsett [1934, 2] has shown that whereas ferric Fe could be recovered quantitatively in trichloroacetic acid extracts of materials containing simple proteins, it could not be recovered from materials containing phosphatides and phosphoproteins. Extraction of ferrous Fe was not so affected. It seems that the absorption of Fe from the alimentary tract is more likely to be hindered by phosphoproteins and phosphatides than by simple proteins.

In previous communications Tompsett [1939, 2, 3] has shown that the absorption of lead is highest on a low calcium diet and least on a high calcium diet and is increased by the addition of mineral acid to the diet. In all probability, Fe absorption is influenced by similar factors.

The dialysis of Fe salts

The object of the present experiments was to determine the factors influencing the dialysis of Fe from organic mixtures and their relation to alimentary absorption.

Parchment thimbles (Whatman) were used as dialysing membranes. In each case 5 ml. of the mixture containing an added Fe salt (1 mg. Fe) were dialysed against 15 ml. of distilled water for 4 hr. At the end of this period the Fe content of the external fluid was estimated by means of thiolacetic acid and NH_3 [Tompsett, 1934, 1, 2].

Egg yolk and egg white suspensions, containing added Fe (ferrous sulphate, iron ammonium citrate, iron alum) were subjected to dialysis. Ferrous Fe dialysed readily, whereas the dialysis of ferric Fe was nil or negligible. Acid peptic digests of egg white and egg yolk, containing added Fe (ferrous sulphate, iron ammonium citrate, iron alum), were then dialysed. Ferrous Fe dialysed readily from all digests but ferric Fe, although dialysing from egg white digests, did not do so from egg yolk digests. These experiments were repeated after neutralization of the digests with $NaHCO_3$. Both ferric and ferrous Fe dialysed readily from neutralized digests of egg white but both showed negligible dialysis from neutralized digests of egg yolk. Ferric Fe was found to dialyse readily from acidified suspensions of undigested egg white.

Experiments were then carried out to determine whether pyrophosphates had any influence on the dialysis of ferric Fe. The pyrophosphate was added as the sodium salt. In acid peptic digests of egg yolk, pyrophosphates had little effect. This might have been due to the rapidity with which pyrophosphates are hydrolysed to orthophosphates by mineral acids. Pyrophosphates increased to

Table 1. *Dialysis of Fe, Cu and Pb*

Fe

1 mg. Fe subjected to dialysis; the figures refer to mg. Fe that have dialysed in 4 hr.

Undigested egg white	
Ferrous sulphate	0·56
Iron alum	0·00
Iron ammonium citrate	0·01
Iron alum + sodium pyrophosphate	0·28
Egg white digested with pepsin, *p*H 4·0	
Ferrous sulphate	0·41
Iron alum	0·41
Iron ammonium citrate	0·38
Peptic digest of egg white neutralized with $NaHCO_3$	
Ferrous sulphate	0·36
Iron alum	0·20
Iron ammonium citrate	0·19
Iron alum + sodium pyrophosphate	0·19
Undigested egg white (acidified), *p*H 4·0	
Iron alum	0·23
Undigested egg yolk	
Ferrous sulphate	0·52
Iron alum	0·00
Iron ammonium citrate	0·00
Iron alum + sodium pyrophosphate	0·26
Egg yolk digested with pepsin, *p*H 4·1	
Ferrous sulphate	0·56
Iron alum	0·03
Iron ammonium citrate	0·03
Iron alum + sodium pyrophosphate	0·03
Peptic digest of egg yolk neutralized with $NaHCO_3$	
Ferrous sulphate	0·03
Iron alum	0·00
Iron alum + sodium pyrophosphate	0·20
Casein digested with pepsin, *p*H 4·2	
Ferrous sulphate	0·39
Iron alum	0·06
Iron ammonium citrate	0·01

Cu

1 mg. Cu subjected to dialysis; the figures refer to mg. Cu that have dialysed in 4 hr.

Undigested egg white	0·00
Acidified undigested egg white (*p*H 4·6)	0·22
Peptic digest of egg white (*p*H 4·2)	0·38
Peptic digest of egg white neutralized with $NaHCO_3$	0·05
Undigested egg yolk	0·04
Peptic digest of egg yolk (*p*H 4·2)	0·38
Peptic digest of egg yolk neutralized with $NaHCO_3$	0·04

Pb

1 mg. Pb subjected to dialysis; the figures refer to mg. Pb that have dialysed in 4 hr.

Undigested egg white	0·00
Acidified undigested egg white (*p*H 4·6)	0·26
Peptic digest of egg white (*p*H 4·2)	0·45
Peptic digest of egg white neutralized with $NaHCO_3$	0·03
Undigested egg yolk	0·03
Peptic digest of egg yolk (*p*H 4·1)	0·48
Peptic digest of egg yolk neutralized with $NaHCO_3$	0·02

a marked extent the dialysis of ferric Fe from neutralized digests of egg yolk, undigested egg yolk and egg white.

Experiments carried out with casein produced results similar to those obtained with egg yolk.

Similar dialysis experiments were carried out using lead acetate and copper sulphate (1 mg. Pb, 1 mg. Cu). The amounts of these metals in the dialysates were determined by methods that have been described already [Tompsett, 1934, 3; 1939, 1]. These indicated that under favourable conditions lead and copper dialysed as readily from egg yolk suspensions as from egg white suspensions.

Examples of the results obtained are shown in Table 1.

Animal experiments

Mice were used as experimental animals. Their total Fe contents were estimated by means of thiolacetic acid and NH_3 [Tompsett, 1934, 1, 2]. An animal was ignited in a silica dish over a Bunsen burner. The ash was dissolved in water containing 5 ml. conc. HCl and the volume made up to 50 ml.; Fe was estimated in an aliquot. A precipitate of Ca phosphate usually developed but this was removed by centrifuging prior to colorimetric analysis. This did not affect the accuracy of the results.

The effect of phosphatides and phosphoproteins upon the absorption of Fe was investigated. Young female mice aged 3 weeks were used. In general, the experiments were carried out under similar conditions to those described in a previous communication [Tompsett, 1939, 2]. Control animals were killed at the commencement of the experiments. The diet, which was of low Ca content, had the following composition: whole wheat flour, 400 g.; corn starch, 325 g.; wheat gluten, 50 g.; olive oil, 40 g.; NaCl, 20 g.; KCl, 15 g.; butter, 15 g. The animals were divided into 2 groups and placed on the following diets:

(1) Diet + 1 ml. egg white per mouse per day.
(2) Diet + 1 ml. egg yolk per mouse per day.

In addition the diets contained supplements of added Fe, at the rate of 0·8 mg. Fe (iron alum or ferrous sulphate) per mouse per day. The duration of the experiments was 14 days. At the end of this period the animals were placed on the diets without added Fe for 4 days to remove unabsorbed Fe from their alimentary tracts; they were then killed and their total Fe contents estimated.

The results are shown in Table 2. It will be seen that phosphatides and phosphoproteins inhibit the absorption of Fe from the alimentary tract, and that similar results were obtained irrespective of whether ferric or ferrous Fe was fed.

Table 2. *Young female mice (aged 3 weeks). Time of experiment 14 days. Fe added: 0·8 mg./mouse/day*

Diet	Form of Fe added	No. of mice in group	Fe mg./100 g./mouse		
			Highest	Lowest	Mean
Control	Iron alum	14	6·76	5·00	**6·11**
Control + egg yolk	,,	12	4·71	3·37	**3·85**
Control + egg white	,,	12	8·16	5·88	**6·80**
Control	$FeSO_4$	6	7·57	4·78	**5·72**
Control + egg yolk	,,	6	5·41	3·94	**4·58**
Control + egg white	,,	6	7·54	6·56	**7·01**

Experiments were carried out in which iron pyrophosphate was the source of Fe. The results, which are not reported in detail, agreed with those obtained with iron alum and ferrous sulphate. This was due probably to the hydrolysis of the pyrophosphate by the acid of the gastric juice.

The influence of the Ca content of the diet upon the absorption of Fe has been investigated. The diets used had the following compositions:

Low Ca diet	g.	High Ca diet	g.
Whole wheat flour	400	Whole wheat flour	700
Casein	100	Whole milk powder	300
Corn starch	325	Marmite	50
Wheat gluten	50		
Olive oil	40		
NaCl	20		
KCl	15		
Butter	15		

The diets, to which were added supplements of Fe (as iron ammonium citrate), were fed at the rate of 2·5 g. per mouse per day. In the first experiments adult male mice were used; the experiments lasted 21 days. At the end of this period the animals were placed on the diets without added Fe for 4 days to remove unabsorbed Fe from their alimentary tracts; they were then killed and their total Fe contents estimated. The experiments were repeated using young female mice aged 3 weeks; the duration of these experiments was 14 days. The results indicate that Fe is absorbed more easily on a low Ca than upon a high Ca diet. The effect of the addition of HCl to the diet upon the absorption of Fe was then investigated. Young female mice aged 3 weeks were used and the duration of the experiment was 14 days. The results indicated that Fe absorption was increased by this means.

In an animal experiment, the subject usually consumes a diet every mouthful of which has the same composition. In man this does not occur. This difference is often neglected when results obtained from animal experiments are interpreted in terms of human nutrition. In view of the fact that the absorption of essential constituents of the diet may be hindered by their mutual inhibition, it seems important for a daily diet to be arranged so that such inhibition is reduced to a minimum. The following experiment was carried out with this aim in view. Young female mice aged 3 weeks were placed on high and low Ca diets on alternate days, together with added Fe (as iron ammonium citrate), for 14 days. At the end of this period, the animals were placed on a high Ca diet alone for 4 days to remove unabsorbed Fe from their alimentary tracts; they were then killed

Table 3. *Influence of Ca content of diet on absorption of Fe given as iron ammonium citrate*

Sex and age	Time in days	Diet	Amount of Fe added mg.	No. of mice in group	Fe, mg./100 g. mouse		
					Highest	Lowest	Mean
Adult male	21	Control	2	5	6·67	6·00	**6·35**
,,	21	High Ca	2	4	6·60	5·83	**6·22**
,,	21	Low Ca	2	4	10·22	8·29	**8·92**
Female, 3 weeks	14	Control	0·8	8	6·80	5·04	**5·53**
,,	14	High Ca	0·8	8	7·77	5·58	**7·27**
,,	14	Low Ca	0·8	8	17·25	8·54	**12·04**
,,	14	High Ca + HCl	0·8	8	13·25	8·75	**11·04**
,,	14	High and low Ca alternate days	0·8	8	5·99	4·38	**5·17**

and their total Fe contents estimated. The results show that the absorption of Fe was very good, but in addition it should be noted that their increases in weight were very much better than in the other experiments.

The results of the above experiments are shown in Table 3.

The reduction of ferric Fe in the alimentary tract and its significance in absorption

The effect of various foodstuffs upon ferric Fe has been examined by means of the $\alpha\alpha'$-dipyridyl reaction [cf. Hill, 1931].

1 mg. ferric Fe (as iron alum) was added to about 1 g. of foodstuff suspended in about 5 ml. of water. After allowing the mixture to stand for 10 min., a little $\alpha\alpha'$-dipyridyl was added. In acid solution (pH 4–5) a number of these substances produced a marked reduction of ferric Fe as evidenced by the production of a red colour. That this was not due to food Fe was indicated by the negative results obtained with controls. At neutral reactions the results were indefinite. Of the substances examined the following were found to be capable of producing a rapid reduction of ferric Fe in acid solutions: whole wheat flour, whole milk powder, wheat gluten, egg white (boiled and unboiled), cows' milk (boiled and unboiled), commercial egg albumin. Casein, glucose, lactose, egg yolk (boiled and unboiled) were found to be without effect upon ferric Fe. Peptic digests of egg white and egg yolk (pH 4–5) were then examined. The former had a marked reducing effect on ferric Fe but the latter was without effect.

The effect on ferrous Fe of those substances which had no reducing action on ferric Fe was then examined. Except in the case of egg yolk no effect was observed. Ferrous Fe allowed to stand in contact with egg yolk became oxidized to the ferric state. This was shown by the fact that when 1 mg. of ferrous Fe (as ferrous sulphate) was added to 5 ml. of egg yolk, the mixture gave no reaction for ferrous iron with $\alpha\alpha'$-dipyridyl at the end of 1 hr. This phenomenon is considered to be due to autoxidation in which the phosphatides play a part.

Ferric Fe has been shown to dialyse readily from acid peptic digests of egg white and from acidified, but not from neutral, egg white. The state of the iron in the dialysates was examined with $\alpha\alpha'$-dipyridyl. It was found that approximately 66 % of the Fe was in the ferrous state and the remainder in the ferric state. The duration of the experiments was long, viz. 4 hr., so that some re-oxidation could have taken place. That reduction had taken place was to be expected. It is quite possible, however, that ferric Fe did dialyse under the conditions in which these experiments were carried out. Ferrous Fe was observed to dialyse readily from egg yolk and from acid peptic digests of egg yolk. That this was possible is due to the fact that autoxidation takes time. It is considered that the inability of ferrous Fe to dialyse from neutralized peptic digests of egg yolk is due primarily to its precipitation as phosphate. Dialysis of ferric Fe from acid peptic digests of casein was almost negligible. This was due to the formation of stable undissociated ferric complexes and the inability of the mixture to reduce ferric Fe.

In an earlier communication it was shown that added ferric Fe could be almost completely recovered in trichloroacetic acid extracts of simple proteins, e.g. egg white [Tompsett, 1934, 1]. These experiments were repeated, the extracts being examined for the presence of ferrous Fe; only traces could be detected, the greater part of the Fe being in the ferric state. This indicates that reduction was not necessary for these extractions. Complexes of ferric Fe with simple proteins are not as stable as those with phosphatides and phosphoproteins.

The evidence that has been presented suggests that ferric Fe as such has very little chance of absorption from the alimentary tract but that ferrous Fe should be absorbed easily. It would appear that ferric Fe can be reduced in the alimentary tract, not in the small intestine as some workers have suggested, but in the stomach. This has been confirmed *in vivo*. Two rats were fed on (*a*) boiled egg white + iron alum, (*b*) boiled egg yolk + iron alum. At the end of 2 hr. they were killed and their stomach and intestinal contents examined for the presence of ferrous Fe with $\alpha\alpha'$-dipyridyl. In the former animal the stomach and intestinal contents gave an immediate and strong reaction for ferrous Fe; no trace of ferrous Fe could be observed in the latter animal.

A clearer insight into the results obtained from the animal experiments can now be obtained. The low absorption of Fe by mice on a diet containing egg yolk was no doubt due to the predominating autoxidation properties of the diet. With egg white in the diet Fe absorption was much higher, owing to the predominating reducing properties of the diet. It also becomes apparent why similar results were obtained when ferrous and ferric Fe were fed with diets containing egg yolk and egg white.

The question naturally arises as to whether the Fe of the egg is available at all, i.e. whether the effect of the white predominates over that of the yolk or *vice versa*. Experiments were carried out to decide this point. Peptic digests of boiled and unboiled whole egg were prepared. At *p*H 4–5, a marked reducing effect was observed. This was confirmed *in vivo* on a rat. These results indicate that the effect of the white predominates over that of the yolk.

Copper

The study of the alimentary absorption of Cu seems to have been neglected. The writer has shown that the absorption of both Fe and Pb from the alimentary tract is influenced by the Ca content of the diet. The addition of acid to the diet increased the absorption both of Fe and of Pb. It seemed very probable that the absorption of Cu would be governed by similar factors and the following experiments were undertaken to investigate this question. Dialysis experiments indicated that the absorption of Cu would probably not be greatly inhibited by the presence of phosphoproteins and phosphatides in the diet as was found to be the case with ferric Fe.

The experiments were carried out in a similar manner to those with Fe. The diets had the same composition and both adult and young female mice were used.

The total Cu contents of the animals were estimated in the ash with sodium diethyldithiocarbamate as described for urine and faeces in a previous communication [Tompsett, 1934, 3].

Table 4. *Influence of diet on absorption of Cu given as Cu sulphate*

Sex and age	Time in days	Diet	Amount of Cu added mg.	No. of mice in group	Cu, mg./100 g. mouse		
					Highest	Lowest	Mean
Adult male	21	Control	2	8	0·17	0·10	**0·14**
,,	21	Low Ca	2	8	0·33	0·21	**0·27**
,,	21	High Ca	2	7	0·18	0·14	**0·16**
,,	21	High Ca + HCl	2	8	0·37	0·21	**0·28**
Female, 3 weeks	14	Control	0·8	5	0·29	0·21	**0·25**
,,	14	Low Ca	0·8	8	0·69	0·30	**0·38**
,,	14	High Ca	0·8	6	0·22	0·18	**0·20**
,,	14	High Ca + HCl	0·8	7	0·42	0·30	**0·36**

The results are shown in Table 4. It will be seen that the absorption of Cu is higher on a low Ca diet than on a high Ca diet and is increased by the addition of acid to the diet. The latter observation indicates the importance of the gastric acidity for the absorption of Cu.

Discussion

It has been shown that certain foodstuffs are capable of reducing ferric Fe to the ferrous state especially at an acid reaction. It is of interest to speculate as to the nature of the substances responsible for this reducing action. Certain vitamins, e.g. ascorbic acid, might prove to be responsible but the writer is inclined to the view that protein is chiefly responsible. More work upon these problems is necessary.

The results obtained in this work suggest that ferric Fe as such stands very little chance of absorption from the alimentary tract. Its absorption would be hindered by the presence of phosphatides and phosphoproteins. Evidence has been produced which shows that ferric Fe can be reduced in the alimentary tract, not in the small intestine as has been suggested, but in the stomach. This reduction is brought about by substances which are common constituents of the diet. On the other hand, the diet may contain substances which produce an opposite effect, i.e. the oxidation of ferrous Fe to the ferric state. Ferrous Fe appears to be the only form to be absorbed from the alimentary tract.

The following factors have been shown to influence the absorption of food Fe from the alimentary tract:

(1) The gastric acidity.
(2) The Ca content of the diet.
(3) The oxidation-reduction potential of the stomach contents.

The gastric acidity is important in two respects: (1) it aids the solution of the Fe; (2) it appears to be necessary for the reducing action of the diet, if any, to take its full effect. The Ca content of the diet has a marked effect. Similar results have been obtained with Pb and suggestions put forward in the report dealing with Pb [Tompsett, 1939, 2] probably apply also to Fe. The composition of the diet determines the state of the ingested Fe, i.e. whether it is in the ferric or ferrous state. In a diet which is predominantly reducing, then, irrespective of whether the Fe was initially in the ferric or ferrous state, its final state will be ferrous and as a result it should be absorbed easily. In a diet which is predominantly oxidizing, the final state of the Fe will be ferric and it is presumably not absorbed. Only in an inert diet will the state of the Fe remain unchanged.

Lack of gastric acidity has been held responsible for the hypochromic anaemia that often occurs in achlorhydria. Anaemia does not always occur in achlorhydria. Even in this condition, the gastric contents have an acid reaction, although this is weak. A certain amount of Fe should pass into solution and it appears that a reducing action might occur. Whether or not the amount of Fe absorbed under such conditions is sufficient to prevent anaemia will depend upon the requirements.

Attempts have been made to determine the minimum amount of Fe necessary in the human diet [Sherman, 1933]. The results obtained have not been particularly convincing. The writer considers that it is impossible to state any particular quantity of Fe as being sufficient since the degree of Fe absorption is dependent on so many factors.

Workers on nutrition usually consider human requirements in terms of the daily intake. The writer has shown that absorption of essential substances may

result in mutual inhibition. A daily diet should be so arranged into meals that mutual inhibition is reduced to a minimum. One could even go a stage further and plan a meal into courses since it is well known that food tends to layer in the stomach so that the digestion and absorption of one course is independent of another. This is of particular importance in children where the requirements are large.

A large number of Fe salts, both ferric and ferrous, have been used in the treatment of hypochromic anaemias. Large doses, especially of the ferric salts, are now usually given. They may be administered on an empty stomach or after a meal. When medicinal Fe is administered after a meal its absorption will be subject to the same factors as that of food Fe. When administered on an empty stomach the conditions of absorption will be different, the constituents of the secretions of the alimentary tract being the only limiting factor. So far these have been neglected, but they must have some influence.

Although ferric Fe appears to be capable of dialysing as the pyrophosphate, this fact appears to be of little physiological importance.

The value of large doses of therapeutic iron, especially of ferric salts, is probably due to the fact that only a part is converted into non-dissociated colloidal complexes. Under such conditions the possibility should not be overlooked that ferric Fe may be absorbed as such.

Absorption of Fe and Cu probably occurs in the upper part of the small intestine where the contents still have a pronounced acid reaction. The reaction of the intestinal contents must exert a very marked influence and this must depend on a number of factors such as the gastric acidity, the base content of the diet and the character of the intestinal secretions. Much more work is still required before we can obtain a clear understanding of Fe absorption.

Summary

1. The dialysis of ferric Fe, but not of ferrous Fe, is inhibited by phosphatides and phosphoproteins.

2. Ferric Fe may be reduced by certain foodstuffs.

3. Ferrous Fe is oxidized readily to the ferric state in the presence of egg yolk, presumably by a process of autoxidation.

4. Ferric Fe does not appear to be capable of absorption from the alimentary tract. Fe, to be absorbed, must be in the ferrous state.

5. Reduction of ferric Fe can occur in the stomach, this being effected by the diet.

6. The absorption of both Fe and Cu from the alimentary tract is influenced by the gastric acidity and the Ca content of the diet.

7. The relation of these factors to human nutrition has been discussed.

I wish to thank Dr A. B. Anderson for his helpful criticism and advice.

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116. THE BODY FATS OF THE PIG

5. COMPONENT GLYCERIDES OF PERINEPHRIC AND OUTER BACK FATS FROM THE SAME ANIMAL

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(*Received 3 May 1940*)

SINCE the glyceride structure of the inner back fat of a pig was investigated by observations on the composition of the fatty acids present after it had been hydrogenated to a number of stages culminating in complete saturation [Hilditch & Stainsby, 1935], a method of more precise determination of the component glycerides present in a solid natural fat has been developed. This procedure, which depends on preliminary resolution of the original fat into several mixtures of less complexity by systematic crystallization from acetone, has already been applied by Hilditch & Paul [1938] to the study of an ox depot (perinephric) fat. We have now taken the opportunity afforded by the possession of sufficient quantities of perinephric and outer back fats from a pig reared on a known diet at the Animal Nutrition Institute, Cambridge, to undertake similar studies of the glycerides present therein.

EXPERIMENTAL

The procedure employed was described in full in the previous communication on an ox depot fat [Hilditch & Paul, 1938], to which reference may be made. The essential data from which the probable component glycerides are deduced will therefore be tabulated in as condensed a form as possible, and description of the methods followed may be confined to the following general statement:

(*a*) The pig fats (*ca.* 1200 g.) were each systematically crystallized from acetone (similarly to the ox depot fat for which a full description was given) and finally grouped into three or four fractions by uniting sub-fractions of similar iodine value.

(*b*) The component acids in each fraction were determined by ester-fractionation.

(*c*) The amounts of fully saturated glycerides present in the two fractions from each fat most sparingly soluble in acetone were determined by oxidation of a portion of each fraction with $KMnO_4$ in acetone solution. When sufficient fully saturated glycerides (10 g. or more) were available, their component acids were determined by ester-fractionation; otherwise, the latter were calculated as mixtures of palmitic and stearic acids from the observed equivalent of the fully saturated glycerides.

(*d*) In the fractions most soluble in acetone (in which the di- and tri-unsaturated glycerides are concentrated), the amount of tri-C_{18} glycerides was determined, in the form of tristearin, by a method which differed slightly from that previously employed. A portion (*ca.* 200–250 g.) of the fraction was completely hydrogenated and the whole of the product separated by crystallization

from anhydrous ether into several sub-fractions, in each of which the component acids were determined by ester-fractionation. The component glycerides in each sub-fraction were then calculated as binary mixtures of tristearin and palmitodistearin, or of palmitodistearin and dipalmitostearin, according to the proportions of palmitic and stearic acid found, whence the tristearin content of the hydrogenated product, and therefrom the proportionate amount of tri-C_{18} glycerides in the whole fat present in the most soluble fractions, were obtained. This procedure, although much more lengthy than the sole determination of the saponification equivalents of the fractions of hydrogenated product separated by crystallization from ether, is believed to give more reliable results.

The whole of the data thus accumulated permit the component glycerides present in each fraction of the original fat to be grouped, according to the proportions of palmitic, stearic and unsaturated C_{18} acids present, into (*a*) dipalmitomono-C_{18} glycerides, palmitodi-C_{18} glycerides, and tri-C_{18} glycerides, and (*b*), fully saturated, mono-unsaturated-disaturated, di-unsaturated-monosaturated, and tri-unsaturated glycerides. From the proportions of these classes of glycerides, the individual components in each fraction of the original fat can be deduced, usually within close limits.

As in the case of the ox depot fats, pig fats contain small quantities of several acids which, for the present purpose, have to be included with one or other of the major component acids. The minor amounts of lauric, myristic, tetradecenoic and hexadecenoic acids are included with palmitic, arachidic (when present) with stearic, and octadecadienoic and unsaturated C_{20-22} acids are similarly included in the "oleic" group. The minor component acids of pig fats, other than octadecadienoic (*ca.* 5 %), together form about 5 % of the total fatty acids, as compared with somewhat more (*ca.* 7 %) in ox fats. As pointed out by Hilditch & Paul [1938], the consequent probable presence of about 15 % of triglycerides containing one of these minor component acyl groups in their molecules does not seriously affect the conclusions reached as regards the distribution of saturated non-C_{18} and stearic acids in the mixed glycerides of the fats, whilst the great preponderance of palmitic acid over the other saturated non-C_{18} acids entails that components defined as "palmito"-glycerides are in fact for the most part compounds of palmitic acid.

The fats studied in the present work were mainly from a single pig (no. 73) which had been reared for 16 weeks from weaning on a low plane of nutrition, and subsequently to 200 lb. live weight on a high plane of nutrition [cf. Hilditch *et al.* 1939]. The perinephric fat used was entirely from this animal, but in order

Table 1. *Component acids of the perinephric and outer back pig fats studied*

	Perinephric (no. 73)		Outer back fats (no. 73)		Outer back fats (no. 74)		Outer back fats Mixture used (calc.)	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Lauric	0·3	0·3	Trace	—	—	—	—	—
Myristic	0·8	1·0	1·1	1·4	1·3	1·6	1·2	1·5
Palmitic	30·4	32·4	28·2	30·2	28·3	30·2	28·3	30·2
Stearic	17·9	17·2	13·5	13·0	11·9	11·5	12·9	12·5
Tetradecenoic	0·1	0·1	0·2	0·2	0·2	0·2	0·2	0·2
Hexadecenoic	1·5	1·7	2·4	2·6	2·7	2·9	2·5	2·7
Oleic	41·2	39·9	47·0	45·4	47·5	46·0	47·2	45·6
Octadecadienoic	5·7	5·6	5·2	5·1	6·0	5·8	5·4	5·3
Unsaturated C_{20-22}	2·1	1·8	2·4	2·1	2·1	1·8	2·3	2·0

to obtain 1200 g. of outer back fat it was necessary to mix 800 g. of fat from pig no. 73 with 400 g. of the outer back fat of pig no. 74, which had been fed throughout on the high plane of nutrition, but which contained almost exactly the same component acids as the outer back fat of pig no. 73. The component acids of the fats studied are given in Table 1.

Glyceride structure of the perinephric fat

Systematic crystallization from acetone yielded finally four groups of fractions, the proportions, characteristics and component acids of which are given in Table 2.

Table 2. *Fractions (A, B, C, D) separated by crystallization of the perinephric fat from acetone*

	A		B		C		D	
Wt. of fraction (g.)	252·8		335·1		65·2		568·8	
I.V.	21·5		36·8		62·1		73·8	
Sap. equiv.	285·4		283·3		285·1		287·2	
Glycerides % (wt.)	20·7		27·4		5·3		46·6	
Glycerides % (mol.)	20·7		27·6		5·3		46·4	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Component acids:								
Lauric	—	—	0·7	1·0	—	—	0·4	0·5
Myristic	—	—	1·2	1·4	2·1	2·5	1·4	1·7
Palmitic	35·6	37·9	34·6	36·6	28·0	29·8	23·5	25·1
Stearic	41·0	39·4	29·1	27·7	5·7	5·5	3·3	3·2
Tetradecenoic	—	—	0·8	0·9	0·5	0·6	0·3	0·4
Hexadecenoic	1·3	1·4	1·0	1·1	3·3	3·5	2·4	2·6
Oleic	20·7	20·0	30·1	28·9	53·4	51·5	57·1	55·5
Octadecadienoic	1·4	1·3	2·5	2·4	5·1	4·9	8·9	8·7
Unsaturated C_{20-22}	—	—	—	—	1·9	1·7	2·7	2·3

The fully saturated glycerides present in the less soluble fractions A and B were determined, together with their component acids, with the results shown in Table 3.

Table 3. *Fully saturated glycerides present in fractions A and B of the perinephric fat*

	A		B	
Wt. of fraction oxidized (g.)	100·8		104·1	
Wt. of crude F.S.G. (g.)	35·3		5·0	
I.V. of crude F.S.G.	0·5		2·6	
Sap. equiv. of crude F.S.G.	283·5		279·1	
Acid value of crude F.S.G.	1·7		1·8	
% (wt.)	33·6		4·3	
% (mol.)	33·8		4·3	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Component acids:				
Myristic	3·5	4·2	—	—
Palmitic	37·8	39·9	67·8	70·2
Stearic	58·7	55·9	32·2	29·8

In the most soluble fraction D the tri-C_{18} glycerides were determined by the procedure outlined on p. 971. The portion (124·7 g.) of the hydrogenated fraction D (198·9 g.) least soluble in ether (62·7 % wt. or 62·3 % mol.) contained the following proportions of component acids: myristic 0·3, palmitic 24·4, stearic 72·1, C_{20-22} 3·1 and oleic 0·1 % (mol.), from which it was estimated to contain

25·9 % (mol.) of tri-C_{18} glycerides (including the C_{20-22} group), corresponding to 16·1 % (mol.) of tri-C_{18} glycerides in fraction D, or to 7·5 % (mol.) in the whole fat. (The more soluble fractions contained respectively 62·0, 42·5 and 46·1 % (mol.) of stearic acid, indicating that tri-C_{18} glycerides were not present therein.)

The probable component glycerides in fractions A, B and D have been deduced (by the reasoning described in previous papers) from the foregoing data, whilst those in the small fraction C (insufficient in quantity for more than component acid determination) have been calculated arbitrarily from the proportions of its component acids. The final results are assembled in Table 4. In the case of fraction D alternative possibilities lead to the limiting values indicated for "oleo"-palmitostearin, palmitodi-"olein", stearodi-"olein" and tri-"olein".

Table 4. *Probable component glycerides (% mol.) present in the constituent fractions and in the whole pig perinephric fat*

	A		B		C	D	Whole fat
Glycerides	20·7		27·6		5·3	46·4	100·0
Component acids (increments):							
Lauric	—		0·3		—	0·2	0·5
Myristic	—		0·4		0·1	0·8	1·3
Palmitic	7·8		10·1		1·6	11·6	31·1
Stearic	8·2		7·6		0·3	1·5	17·6
Tetradecenoic	—		0·2		Trace	0·2	0·4
Hexadecenoic	0·3		0·3		0·2	1·2	2·0
Oleic	4·1		8·0		2·7	25·8	40·6
Octadecadienoic	0·3		0·7		0·3	4·0	5·3
Unsaturated C_{20-22}	—		—		0·1	1·1	1·2
	F.S.G.	Mixed	F.S.G.	Mixed			
Groups of component acids (increments):							
C_{16} (+C_{14}, C_{12})	3·1	5·0	1·1	10·2	1·9	14·0	35·3
Stearic	3·9	4·3	0·5	7·1	0·3	1·5	17·6
"Oleic" (+C_{20-22})	—	4·4	—	8·7	3·1	30·9	47·1
Groups of component glycerides (increments):							
Tripalmitin	—	—	0·1	—	—	—	0·1
Dipalmitomono-C_{18}	2·3	1·4	1·5	4·6	0·4	3·1	13·3
Palmitodi-C_{18}	4·7	12·3	—	21·4	4·9	35·8	79·1
Tri-C_{18}	—	—	—	—	—	7·5	7·5
Fully saturated	7·0	0·5	1·6	—	—	—	9·1
Mono-"oleo"-disaturated	—	13·2	—	26·0	1·4	7·6–3·1	48·2–43·7
Di-"oleo"-monosaturated	—	—	—	—	3·9	31·3–40·3	35·2–44·2
Tri-"olein"	—	—	—	—	—	7·5–3·0	7·5–3·0
	Probable component glycerides						
Fully saturated (9·1 %):							
Tripalmitin	—	—	0·1	—	—	—	0·1
Dipalmitostearin	2·3	0·5	1·5	—	—	—	4·3
Palmitodistearin	4·7	—	—	—	—	—	4·7
Mono-"oleo"-disaturated (48·2–43·7 %):							
"Oleo"-dipalmitin	—	0·9	—	4·6	0·4	3·1	9·0
"Oleo"-palmitostearin	—	12·3	—	21·4	1·0	4·5–Nil	39·2–34·7
"Oleo"-distearin	—	—	—	—	—	—	—
Di-"oleo"-monosaturated (35·2–44·2 %):							
Palmitodi-"olein"	—	—	—	—	3·9	31·3–35·8	35·2–39·7
Stearodi-"olein"	—	—	—	—	—	Nil–4·5	Nil–4·5
Tri-"olein" (7·5–3·0 %)	—	—	—	—	—	7·5–3·0	7·5–3·0

Glyceride structure of the outer back fat

Three groups of fractions were finally obtained by systematic crystallization of the outer back fat (67 % from pig no. 73, and 33 % from pig no. 74) from acetone; these had the characteristics shown in Table 5.

Table 5. *Fractions (A, B, C) separated by crystallization of the outer back fat from acetone*

	A		B		C	
Wt. of fraction (g.)	177·3		243·0		780·7	
I.V.	25·0		36·2		74·1	
Sap. equiv.	285·9		285·9		286·9	
Glycerides % (wt.)	14·8		20·2		65·0	
Glycerides % (mol.)	14·8		20·3		64·9	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Component acids:						
Myristic	—	—	0·1	0·1	1·7	2·0
Palmitic	34·8	37·1	34·2	36·4	23·2	24·8
Stearic	38·6	37·2	30·4	29·2	3·7	3·6
Tetradecenoic	—	—	—	—	0·3	0·4
Hexadecenoic	0·7	0·8	1·7	1·9	2·8	3·1
Oleic	22·5	21·8	28·2	27·3	55·7	54·1
Octadecadienoic	2·2	2·1	4·0	3·9	9·5	9·3
Unsaturated C_{20-22}	1·2	1·0	1·4	1·2	3·1	2·7

The fully saturated glycerides (and their component acids) present in the less soluble fractions A and B were found to be as indicated in Table 6.

Table 6. *Fully saturated glycerides present in fractions A and B of the outer back fat*

	A		B	
Wt. of fraction oxidized (g.)	101·6		133·4	
Wt. of crude F.S.G. (g.)	31·4		5·7	
I.V. of crude F.S.G.	0·5		1·1	
Sap. equiv. of crude F.S.G.	281·1		271·3	
Acid value of crude F.S.G.	1·9		1·7	
% (wt.)	29·7		4·0	
% (mol.)	30·2		4·2	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Component acids:				
Myristic	6·5	7·6	—	—
Palmitic	39·0	41·0	91·4	92·2
Stearic	54·5	51·4	8·6	7·8

A portion of the most soluble fraction C (251·4 g.), after complete hydrogenation, yielded three fractions by crystallization from ether, the component acids in the least soluble of which (177·4 g.; i.e. 70·6 % wt. or 70·4 % mol.) were myristic 1·1, palmitic 25·1, stearic 71·3, C_{20-22} 2·4, and oleic 0·1 % (mol.). (The more soluble fractions contained 60·5 and 56·2 % (mol.) of stearic acid, indicating that tri-C_{18} glycerides were not present therein.) The data correspond with 21·4 % (mol.) of tri-C_{18} glycerides (including the C_{20-22} group) in the least soluble fraction of the hydrogenated portion C, and, accordingly, with 15·0 % (mol.) of tri-C_{18} glycerides in the whole fraction C, and with 9·8 % (mol.) in the whole outer back fat.

Consideration of all the data then lead to the final estimates of component glycerides in each of the fractions A, B and C given in Table 7; as in the previous

case, alternative possibilities for some of the components in the most soluble fraction lead to possible values varying within the limits of a few units per cent. for "oleo"-palmitostearin, palmitodi-"olein", stearodi-"olein" and tri-"olein".

Table 7. *Probable component glycerides (% mol.) present in the constituent fractions and in the whole pig outer back fat*

	A		B		C	Whole fat
Glycerides	14·8		20·3		64·9	100·0
Component acids (increments):						
Myristic	—		Trace		1·3	1·3
Palmitic	5·5		7·4		16·1	29·0
Stearic	5·5		5·9		2·4	13·8
Tetradecenoic	—		—		0·2	0·2
Hexadecenoic	0·1		0·4		2·0	2·5
Oleic	3·2		5·6		35·1	43·9
Octadecadienoic	0·3		0·8		6·1	7·2
Unsaturated C_{20-22}	0·2		0·2		1·7	2·1
	F.S.G.	Mixed	F.S.G.	Mixed		
Groups of component acids (increments):						
C_{16} (+C_{14})	2·2	3·4	0·8	7·0	19·6	33·0
Stearic	2·3	3·2	0·1	5·8	2·4	13·8
"Oleic" (+C_{20-22})	—	3·7	—	6·6	42·9	53·2
Groups of component glycerides (increments):						
Tripalmitin	—	—	0·6	—	—	0·6
Dipalmitomono-C_{18}	2·1	—	0·3	1·6	3·8	7·8
Palmitodi-C_{18}	2·4	10·3	—	17·8	51·3	81·8
Tri-C_{18}	—	—	—	—	9·8	9·8
Fully saturated	4·5	—	0·9	—	—	5·4
Mono-"oleo"-disaturated	—	9·6	—	19·0	10·8–3·8	39·4–32·4
Di-"oleo"-monosaturated	—	0·7	—	0·4	44·3–58·3	45·4–59·4
Tri-"olein"	—	—	—	—	9·8–2·8	9·8–2·8
Probable component glycerides						
Fully saturated (5·4%):						
Tripalmitin	—	—	0·6	—	—	0·6
Dipalmitostearin	2·1	—	0·3	—	—	2·4
Palmitodistearin	2·4	—	—	—	—	2·4
Mono-"oleo"-disaturated (39·4–32·4%):						
"Oleo"-dipalmitin	—	—	—	1·6	3·8	5·4
"Oleo"-palmitostearin	—	9·6	—	17·4	7·0–Nil	34·0–27·0
"Oleo"-distearin	—	—	—	—	—	—
Di-"oleo"-monosaturated (45·4–59·4%):						
Palmitodi-"olein"	—	0·7	—	0·4	44·3–51·3	45·4–52·4
Stearodi-"olein"	—	—	—	—	Nil–7·0	Nil–7·0
Tri-"olein" (9·8–2·8%)	—	—	—	—	9·8–2·8	9·8–2·8

Discussion

The present data offer for the first time an opportunity to compare the component glycerides of fats of different mean unsaturation present in different fat depots of one and the same animal. They thus form a useful basis for further consideration of the suggestion [Hilditch & Sleightholme, 1931; Banks & Hilditch, 1932; Hilditch, 1937, 1, 2; Hilditch & Paul, 1938] that stearo-glycerides in stearic-rich animal depot fats have resulted from saturation of preformed oleo-glycerides. At the same time, the procedure used in the present work has defined the glyceride components of the pig fats within much closer limits than was possible by earlier methods, such as that of progressive hydrogenation employed by Hilditch & Stainsby [1935]. Both these points are illustrated in

Table 8, in which are given (to the nearest unit) the component glycerides observed in the present work on pig perinephric and outer back fats, by Hilditch & Paul in ox depot fat, and in the earlier work of Hilditch & Stainsby on a pig inner back fat; the stearic acid content of the total fatty acids from each fat is also given.

Table 8. *Component glycerides of pig and ox depot fats* (% *mol.*)

Fat	(Hilditch & Stainsby [1935]) Pig, inner back	Present investigation: Pig, outer back	Present investigation: Pig, perinephric	(Hilditch & Paul [1938]) Ox, perinephric
% Stearic acid in total acids	14·4	13·8	17·6	22·6
Component glycerides				
Fully saturated:				
Tripalmitin	—	1	—	3
Dipalmitostearin	3	2	4	8
Palmitodistearin	2	2	5	6
Mono-"oleo"-disaturated:				
"Oleo"-dipalmitin	2	5	9	15
"Oleo"-palmitostearin	36–21	34–27	39–34	35–32
"Oleo"-distearin	—	—	—	2
Di-"oleo"-monosaturated:				
Palmitodi-"olein"	42–57	46–53	35–40	20–23
Stearodi-"olein"	0–15	0–7	0–5	8–11
Tri-"oleins"	15–0	10–3	8–3	3–0

Comparison of the first column in Table 8 with the remaining three columns (the data in which are based on our current procedure of preliminary crystallization of the fats from acetone) shows that the possible limits for the proportions of "oleo"-palmitostearin, palmitodi-"olein", "stearodi-"olein" and tri-"oleins" have been considerably narrowed. The remaining uncertainty, amounting to 3–7 units %, is due entirely to the difficulty, still inherent in the experimental method, of distinguishing between stearodi-"olein" and tri-"oleins" in the more liquid and soluble fractions of the fat. The possible limits are nevertheless sufficiently near in each case to justify the claim that a practically complete statement of the component glycerides in animal depot fats can now be given.

In the perinephric and outer back fats of the pig studied in the present work "oleo"-palmitostearins and palmitodi-"oleins" are the chief components, and in each case amount together to about 75–80 % of the whole fats. In the more unsaturated outer back fat, palmitodi-"oleins" form about half of the fat and considerably exceed in amount the "oleo"-palmitostearins; but in the perinephric fat the proportion of each group is of the same order (whilst in the more saturated ox depot fat there is more "oleo"-palmitostearin than palmitodi-"olein"). Small amounts (3 %) of tri-unsaturated C_{18} glycerides are definitely present in both pig fats, and the proportion may be greater according to whether stearodi-"oleins" are also present; the total amount of these tri-C_{18} glycerides is of the order of 8–10 % in each fat. The remaining glyceride components, although significant, are individually of a minor order.

The present data, in conjunction with those for the ox depot fat, give some further insight into the possible hydrogenation mechanism of the production of animal depot glycerides which are relatively rich in combined stearic acid. The suggestion that hydrogenation of preformed oleo-glycerides in the animal

may be involved was originally based [Banks & Hilditch, 1932] on the facts that abnormal increase in the content of fully saturated glycerides accompanies increasing proportions of stearic acid in the total fatty acids of these animal depot fats, whilst the content of the other major component saturated acid, palmitic acid, remains approximately constant at about 30 ± 3 % (mol.) [Hilditch & Longenecker, 1937]. These phenomena are well illustrated by the figures for the present pig outer back and perinephric fats, and the ox perinephric fat, in which (whilst the palmitic acid contents are respectively 29·0, 31·1 and 32·2 % mol.) the stearic acid contents steadily increase (respectively 13·8, 17·6 and 22·6 % mol.) and correspondingly the fully saturated glycerides were found to amount to 5, 9 and 17 % (mol.) of the whole fat.

The present study enables us to show in addition, however, that there is a corresponding relationship between the palmitodiolein, oleopalmitostearin and palmitodistearin groups in each fat; and, to a less marked extent, between the proportions of oleodipalmitin and dipalmitostearin in each fat. In the most unsaturated of the three fats (pig outer back fat), palmitodioleins exceed oleopalmitostearins in amount; in the pig perinephric fat the amount of each is approximately the same, and in the ox perinephric fat (which contains nearly 23 % of stearic acid), palmitodioleins are present in much less proportion than the oleopalmitostearins. The major component glycerides (75–80 %) of the fats, therefore, consist of palmitodi-C_{18} glycerides, the relative proportions of oleopalmitostearin and palmitodiolein in which (like the fully saturated glyceride contents of the fats) run parallel with the stearic acid contents of the whole fats. This of course is fully consistent with, and additional evidence in favour of, the hypothesis of saturation of preformed oleo-glycerides which we have previously put forward.

At the same time, whilst this is the most prominent feature of these comparative studies of ox and pig depot glyceride structure, the detailed data available for the two pig fats now studied and for the ox depot fat disclose a subordinate phenomenon. With increasing general saturation (i.e. as already shown, increase in stearic at the expense of oleic acid in the fats as a whole) there is a minor, but definite increase in the amount of dipalmitomono-C_{18} glycerides (in the three fats, respectively, these amount to 7, 13 and 23 % mol.), although the content of tri-C_{18} glycerides does not alter significantly. This may in part be correlated with the slight increase (with decreasing mean unsaturation of the whole fats) in palmitic acid content within the range of 30 ± 3 % (mol.), but, in the ox fat, is also due to the higher proportions of the minor component, myristic acid (which is grouped with palmitic acid in the present experimental studies of glyceride structure). The present data, however, point definitely to the presence of subordinate changes in glyceride structure, following decrease in general unsaturation of the depot fats, which stand outside the conception of hydrogenation of preformed oleo-glycerides; the latter hypothesis, however, satisfactorily accounts for the main features which are characteristic of the glyceride structure of stearic-rich animal depot fats.

Finally, it may be recalled that, in the ox depot fat, the proportions of "oleo"-dipalmitin, "oleo"-palmitostearin, palmitodi-"olein" and stearodi-"olein" obtained by an arithmetical calculation, based (after allowance for the fully saturated glycerides present) upon proportionate division of the "oleic" acid between the palmitic and stearic acids present in the form of mixed "oleo"-saturated glycerides, were extremely close to those actually observed. In the two pig fats now under consideration, a similar calculation leads in each case to values which differ somewhat widely from the observed figures for "oleo"-

dipalmitin, "oleo"-palmitostearin and stearodi-"olein", although they are closer to the latter in the case of palmitodi-"olein" (calculated, outer back fat 43%, perinephric fat 32% mol.). The accordance in these "calculated" and observed figures in the case of the ox depot fat glycerides may therefore have been merely a coincidence.

Summary

Specimens of outer back and perinephric fats from the same pig have been studied after resolution into several fractions of varying degrees of solubility in acetone, with results leading to an approximate statement of the proportions of each of the major component mixed glycerides present in each fat. The approximate molar percentages of the main components were:

	Outer back fat % (mol.)	Perinephric fat % (mol.)
Palmitodi-"olein"	46–53	35–40
"Oleo"-palmitostearin	34–27	39–34
"Oleo"-dipalmitin	5	9
Stearodi-"olein"	0–7	0–5
Tri-"oleins"	10–3	8–3
Fully saturated glycerides	5	9

The proportions of the respective groups of tri-"olein", palmitodi-"olein", "oleo"-palmitostearin, and of the fully saturated glycerides in each pig fat, together with those in an ox perinephric fat previously studied [Hilditch & Paul, 1938], form a series which strongly supports the hypothesis that stearo-glycerides in these animal depot fats have resulted from saturation of preformed oleo-glycerides. Concurrently, the three groups of observations disclose a subordinate effect not connected with a hydrogenation mechanism, namely, a gradual increase in the proportions of dipalmito-glycerides as the general degree of saturation of the depot fats becomes more pronounced.

We offer cordial thanks to Dr J. Hammond of the Animal Nutrition Institute, School of Agriculture, Cambridge, to whom we owe the experimental material used in this investigation. The work forms part of a programme undertaken by the Food Investigation Board, and we thank the Department of Scientific and Industrial Research for permission to publish these results.

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117. ON THE SYNTHESIS OF COCARBOXYLASE

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(*Received 15 May 1940*)

WEIJLARD & TAUBER [1938] have described a method for the synthesis of cocarboxylase from aneurin which is important not only because it renders this substance easily accessible, but also because of the general interest attached to the synthesis of a primary ester of pyrophosphoric acid in a pure state, a problem which has never been satisfactorily solved before.

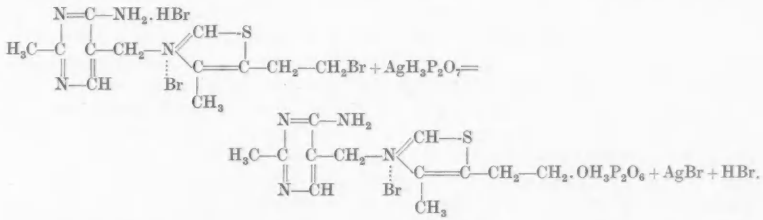
Weijlard & Tauber only described their method briefly without studying or discussing the mechanism of the synthesis or the progress of the purification. Because a proper understanding of the reactions involved was lacking, great difficulties were encountered by the writer when he first tried to repeat Weijlard & Tauber's experiments with smaller quantities of aneurin than those used by them. The product finally isolated, although catalytically active, had a low P content; it could be shown to contain an admixture of 50–70% of aneurin monophosphate from which it could not be separated by repeated recrystallizations. Similar difficulties were apparently met with in the Oxford Laboratory (personal communication from Mr L. A. Stocken).

These failures led to a detailed investigation of the synthesis of cocarboxylase. As a result an alternative method of synthesis has been worked out which is probably capable of a wider application to the synthesis of primary esters of pyrophosphoric acid. In addition, a number of observations have been made which, it is hoped, will contribute to the understanding of the mechanism of this synthesis in particular and of the synthesis of pyrophosphoric esters in general.

After it had been found that by the method of Weijlard & Tauber a large proportion of the monophosphate was obtained, it was assumed that this was the result of the hydrolysis of a primary pyrophosphate originally formed and that the pyrophosphate present in the end product had by chance escaped this hydrolysis. Since the isolation and purification was carried out with the greatest possible speed and under the mildest possible conditions of temperature and *p*H, the hydrolysis could only have occurred during the actual phosphorylation. The esterification of aneurin involves the liberation of 1 mol. of water and it was thought possible that this might lead to an immediate hydrolysis of a part of the pyrophosphate. This view was confirmed by the opinion expressed by Wagner-Jauregg & Griesshaber [1937, 2] that the method of preparing organic pyrophosphoric acids by the action of P_2O_5 is liable to fail in all those cases in which water can be formed.

Consequently a method was devised where the liberation of water was entirely eliminated. This was attained by the interaction of the Br-derivative of aneurin (3-(2-methyl-4-aminopyrimidyl-5-methyl)-4-methyl-5-bromoethyl-

thiazolium bromide hydrobromide; henceforth abbreviated to "bromo-aneurin") with silver pyrophosphate.



Substitution of the OH group of aneurin by Cl occurs when aneurin is heated with conc. HCl at 150° [Buchman & Williams, 1935], but at the same time the NH_2 group in position 4 of the pyrimidine ring is replaced by OH. It was thought likely that the hydrolysis of the NH_2 group could be avoided by working under strictly anhydrous conditions. The vitamin was therefore heated in sealed tubes with HBr in acetic acid for 3 hr. at 130°. The yield of bromo-aneurin amounted to 60 % on the average. The conditions mentioned were found to give the best results. If the time of heating was shortened or the temperature lowered, a considerable proportion of aneurin was recovered unchanged, whereas under more drastic conditions disruption of the molecule became marked.

Bromo-aneurin crystallizes in long felted needles. It melts at 234° (uncorr.) with decomposition. The thiochrome reaction is positive indicating an intact 4′-amino group.

From this compound cocarboxylase was prepared by interaction with silver pyrophosphate. In the above equation the reaction is assumed to take place between bromo-aneurin and monosilver pyrophosphate. Attempts were made to prepare this salt in a pure state. Silver pyrophosphate was dissolved in an excess of hot pyrophosphoric acid. The mixture was then treated with acetone, an insoluble residue was filtered off and the clear acetone solution was precipitated by the addition of a further quantity of acetone. The precipitate had a composition which corresponded roughly to a salt of the formula $Ag_3H_5P_4O_{14}$, i.e. the ratio of Ag : P was 1·5 : 2. Several variations were tried, but it was never possible to obtain a compound with a smaller ratio of Ag : P. According to Wagner-Jauregg [1936] acridine pyrophosphate also contains 3 mol. of acridine to 4 atoms of P.

The phosphorylation of bromo-aneurin was carried out by heating it with a solution of silver pyrophosphate in excess pyrophosphoric acid. Determination of the AgBr formed showed that the reaction was complete after heating at 100° for 40 min.

It soon became apparent that in this method, too, a considerable amount of monophosphate was formed, although it was possible to eliminate entirely one danger point where in Weijlard & Tauber's method a secondary hydrolysis might conceivably have occurred. In this method the melt containing a large excess of pyrophosphoric acid is dissolved in a small amount of water. The resulting solution will have a high concentration of H^+. We have replaced this step by an extraction of the excess pyrophosphoric acid with acetone. The insoluble residue consists of AgBr and aneurin phosphates and can be treated with water without any danger of hydrolysis of the primary pyrophosphates present.

The aqueous extract was now freed from Ag^+ and inorganic pyrophosphate was removed with $Ba(OH)_2$ until no further precipitate was formed. If the solution was now analysed it was repeatedly found that it contained acid-hydrolysable P and total organic P in a ratio of exactly 1 : 4. This result was obtained with such regularity that it could not be a coincidence, but must have been due to the mechanism of the reaction. It was concluded that in the reaction between bromo-aneurin and silver pyrophosphate for each molecule of primary pyrophosphoric ester 1 mol. of the secondary symmetrical pyrophosphoric ester was formed.

If such a mechanism was assumed the amount of acid-hydrolysable P and total organic P accounted for almost 100 % phosphorylation of bromo-aneurin. Estimation of the catalytic activity showed that the whole of the primary pyrophosphate present was cocarboxylase.

Naturally these findings were correlated with the peculiar composition of the acid silver pyrophosphate $Ag_3H_5P_4O_{14}$ which can be regarded as a mixture of equal amounts of the primary and secondary pyrophosphates. It was thought that it might be possible to increase the yield of the primary ester if the presence of the secondary salt could be eliminated. The only monobasic salt of pyrophosphoric acid known is the monosodium pyrophosphate, $NaH_3P_2O_7$, described by Giran [1903]. Since Giran's method is cumbersome and requires special equipment, a similar procedure was adopted as in the case of the acid silver pyrophosphate. The product isolated had indeed a ratio of Na : P of 1 : 2, as required for $NaH_3P_2O_7$.

This seemed to indicate that in a solution of sodium pyrophosphate in excess pyrophosphoric acid only the monobasic salt is present. When bromo-aneurin was heated with such a solution, the degree of phosphorylation was very small and amounted only to about 3 % although the time of heating was extended to 2 hr. and the temperature raised to 120°. Again the ratio of acid-hydrolysable P to total organic P was 1 : 4. It is obvious therefore that the secondary symmetrical pyrophosphoric ester was formed even under conditions where the absence of a secondary salt of pyrophosphoric acid can be assumed.

At this stage it was decided to reinvestigate the method of Weijlard & Tauber from the same points of view. In the one experiment performed phosphorylation amounted to 100 % of the aneurin used and the ratio of acid-hydrolysable to total organic P was exactly 1 : 4. The enzymically estimated cocarboxylase however accounted for only 50 % of the acid-hydrolysable P. The higher temperature employed in this method (15 min. at 155°) had apparently brought about a considerable destruction of the molecule.

In this connexion it is interesting to note that ethyl metaphosphate combines with water in such a way that from 4 mol. 1 mol. of symmetrical and 1 mol. of asymmetrical diethylpyrophosphate are formed [Langheld *et al.* 1912]. Accordingly, Wagner-Jauregg & Griesshaber [1937, 1] only found 1/5–1/4 of the total P of ethyl metaphosphate in an easily hydrolysable form. The same authors [1937, 2], when attempting to prepare the primary pyrophosphoric ester of lactic acid by the action of pyrophosphoryl chloride, found that not only the desired primary asymmetrical ester, but also the secondary symmetrical ester was formed.

From our own observations and the data in the literature it is perhaps permissible to conclude for non-enzymic reactions that, as a general rule, wherever a primary asymmetrical ester of pyrophosphoric acid is formed, a secondary symmetrical ester is formed simultaneously. The reasons are probably to be sought in the configuration of the pyrophosphoric acid molecule.

It remained to work out a rational method for the separation of the two esters. Weijlard & Tauber apparently succeeded in preparing the pure pyrophosphate, but it is not clear from their paper by which step of their purification procedure a separation is achieved. It must be attributed to this lack of knowledge that our earlier attempts to adapt their method to a smaller scale had failed.

An analysis of the various steps of the purification showed that neither the precipitation of the Ag salts nor the recrystallization from aqueous acetone or alcohol are in themselves capable of effecting a separation. The separation is based on the fact that the phosphotungstate of aneurin monophosphate has a much higher solubility in aqueous acetone than the phosphotungstate of cocarboxylase. This difference of solubilities is much less marked in the absence of phosphotungstic acid. The increase of the solubility of aneurin monophosphate in aqueous acetone brought about by phosphotungstic acid can easily be demonstrated by the following simple experiment:

100 mg. aneurin monophosphate were dissolved in 10 ml. $N/10$ HCl. On the addition of 100 ml. acetone a milky turbidity appeared which rapidly increased in density. When now 2 g. phosphotungstic acid, dissolved in a little acetone, were added the precipitate immediately dissolved again.

If the mixed phosphotungstates are dissolved in a small amount of aqueous acetone (2 vol. acetone + 1 vol. $N/10$ HCl) the addition of 10 times as much acetone as the $N/10$ HCl which is contained in the amount of aqueous acetone used will precipitate only the pyrophosphate. The monophosphate is precipitated only after the addition of a further 10 vol. acetone. A similar behaviour is shown by the reineckates.

Weijlard & Tauber use a rather tedious procedure of successive extractions with acetone and $N/10$ HCl for decomposing the phosphotungstate precipitate. In their method of decomposing the phosphotungstates there is a factor of uncertainty, i.e. the amount of water retained by the precipitate, which will depend on the bulk and depth of the precipitate and also on the efficiency of centrifuging. Our failure to repeat Weijlard & Tauber's experiments may perhaps be explained on the assumption that when the method was adapted to a smaller scale, the ensuing phosphotungstate precipitate was less bulky, shallower, and more closely packed down on centrifuging. The amount of water it contained was insufficient to retain in solution the phosphotungstate of the monophosphate after the addition of acetone, whereas this must have been the case in the experiments of Weijlard & Tauber. This element of uncertainty has been largely eliminated in the modification here proposed, where the entire precipitate is dissolved in a small amount of aqueous acetone before fractionally precipitating with acetone.

In the method of purification finally adopted, the phosphotungstate precipitation is carried out twice. The silver precipitation is retained as a precaution though it could probably be omitted. After recrystallization from aqueous acetone and alcohol a product was obtained which crystallized readily and gave the correct analytical figures. The yield was 50–60 % of the cocarboxylase estimated in the original solution of the phosphorylation products. The yield from bromo-aneurin is about 17 % and the overall yield from aneurin about 10 %. This is about the same yield as in Weijlard & Tauber's method (8–9 %). Whereas in this method the losses chiefly occur during the actual phosphorylation owing to thermal destruction, this is counterbalanced in the method here described by the losses occurring during the preparation of the bromo-aneurin. If it were possible to prepare this substance in a better yield either from aneurin itself or by a method similar to one of those used for the synthesis of aneurin,

it might be possible to improve the overall yield considerably. Until then most workers will prefer to use the method of Weijlard & Tauber because of its greater simplicity, with certain modifications in the procedure of purification as described in the experimental part.

The P figures tend to be a little too high in the product prepared by the method of Weijlard & Tauber, probably owing to a slight contamination with thiazole pyrophosphate. Through the courtesy of Dr Weijlard I have been supplied with a sample of Merck's cocarboxylase and I have thus been able to compare this preparation with those I had prepared myself. Table 1 shows the

Table 1. *Comparison of P contents and catalytic activities of various cocarboxylase preparations*

Substance	A	B	C	D	Calculated for cocarboxylase
Total organic P (%)	13·72	13·70	13·18	12·92	12·93
Acid-hydrolysable P (%)	6·64	6·26	6·38	6·50	6·46
μl. CO_2 developed after 15 min. with					
1 μg.	56·5	64	—	63·5	—
3 μg.	185·5	178	—	184	—
10 μg.	420	466	—	420	—

result of this comparison with regard to P analyses and catalytic activity. Substance A is Merck's cocarboxylase, substance B is a preparation made by the method of Weijlard & Tauber using the modified purification procedure, substance C was prepared from bromo-aneurin and silver pyrophosphate and analysed at the same stage of purification as substance B, substance D was prepared from substance C by two further recrystallizations from aqueous alcohol. The acid-hydrolysable P was estimated after 20 min. heating with *N* acid in a boiling water bath. The catalytic activity was estimated with the soluble enzyme preparation recently described [Weil-Malherbe, 1939]. Each manometer cup contained 1 mg. protein, *M*/30 phosphate buffer, *p*H 6·2, *M*/30 pyruvate, 0·1 mg. Mg^{++}, 0·1 mg. Mn^{++} in a total volume of 3 ml.

It appears that the catalytic activities of the three preparations are practically identical. Mr L. A. Stocken kindly carried out a comparison of the catalytic activity of substance D with that of pure natural cocarboxylase isolated from yeast by Prof. Lohmann and he reported, rather surprisingly, that the synthetic cocarboxylase had only 60 % of the activity of natural cocarboxylase. There is no doubt that substance D is a reasonably pure product as shown not only by the P analyses, but also by the figures for C, H and N (see Experimental Part). The low catalytic activity might suggest that the synthetic product exists in two isomeric modifications though it is hard to see what kind of isomerism there could be.

From the mother liquors the second phosphoric ester was isolated. It had a P content of 7·5–7·6 % and contained no acid-hydrolysable P. Although it is very probable that the substance formed during the phosphorylation is the secondary symmetrical pyrophosphate, it might have been hydrolysed during the isolation. That such had been the case could be shown by the titration of the product, before and after heating with *N* acid for 2 hr. in a boiling water bath. This treatment would have hydrolysed any persisting pyrophosphate group with the result that a new acidic group would have been formed for each aneurin radical. Actually, exactly two acidic groups were titrated against phenolphthalein both before and after heating with acid. This is the result to be expected for aneurin monophosphate, whereas only one acidic group per aneurin radical would have been titrated in the case of the secondary symmetrical

pyrophosphate. The latter therefore seems to be an unstable compound which is hydrolysed even under the mild conditions prevailing during the isolation.

Reference has been made to the fact that of the cocarboxylase formed only 50–60 % could be isolated in a pure state. Much time and labour has been spent in attempts to improve this yield, but without success. Some observations have been made, however, which it may be worth while to put on record. Our experiments were done along two lines: first the precipitation of the two esters by the salts of various heavy metals was studied in the hope of finding a more efficient separation. According to Lohmann & Schuster [1937] the only insoluble salt of cocarboxylase is the Ag salt. This is not correct since a number of other salts of heavy metals were found to precipitate cocarboxylase completely from 0·0025 *M* solution at *p*H 7. The following ions had this effect: Hg^{++} (mercuric acetate), Bi^{+++} (bismuth nitrate), Cu^{+} (cuprous chloride), Pb^{++} (lead acetate), UO_2^{++} (uranyl acetate), V^{++} (vanadium chloride). The precipitation became complete only at a *p*H of 7 or slightly above. But in all cases the monophosphate was precipitated as well, at least partly. In some experiments a certain fractionation could be obtained, especially with the Hg^{++} and Bi^{+++} salts. But the result was not easily reproducible, probably owing to small differences of *p*H.

Secondly, attempts were made to use organic bases, such as strychnine, brucine and acridine, for the preparation of salts of cocarboxylase which it was hoped might have a greater tendency to crystallization. Acridine has been recommended by Wagner-Jauregg [1936] as specially suitable for the preparation of crystallized derivatives of phosphoric esters. In the case of the aneurin phosphates it was however not possible to prepare these salts. No crystallization occurred from the aqueous solutions and from dilute alcohol the unchanged aneurin phosphate crystallized out. Equally unsuccessful was the use of acridine methochloride.

Experimental Part

Phosphorus estimations. Phosphorus was estimated colorimetrically by the method of Berenblum & Chain [1938]. If ammonium molybdate is added to a solution containing aneurin phosphates a voluminous precipitate is formed. If the blue colour is developed in the presence of the precipitate it is very firmly adsorbed on its surface. With the method of Berenblum & Chain this complication does not arise since phosphomolybdic acid is extracted before the reducing agent is added. This method was found to be very reliable and satisfactory in other respects too. Its great sensitivity enabled the P-estimation to be carried out with quantities of 0·5 mg. cocarboxylase and 0·8–1·0 mg. monophosphate.

Total organic P was estimated after digestion with 60 % perchloric acid, pyro-P after heating with *N* H_2SO_4 in a boiling water bath for 20 min.

Pyrophosphoric acid. The commercial product (B.D.H.) was found still to contain a large proportion of orthophosphoric acid. It was rapidly heated to 300° and kept there for a few minutes. After cooling, a neutralized solution gave a pure white precipitate with $AgNO_3$; an acid solution did not coagulate a solution of egg albumin. P found: 35·05 %, calc. for $H_4P_2O_7$: 34·84 %.

Preparation of $Ag_2H_2P_2O_7$. 17 g. $AgNO_3$ were dissolved in 100 ml. water and 100 ml. *N* NaOH were added. The precipitate was filtered and well washed. 80 g. of pyrophosphoric acid were dissolved in 400 ml. water at 0°. The silver oxide precipitate was stirred into this solution where it dissolved almost completely. After filtering, 3 vol. alcohol were added. The precipitate was collected

on a Büchner funnel, washed with alcohol, acetone and ether, and dried. Analysis showed that it consisted of a mixture of 23% $Ag_4P_2O_7$ and 77% $Ag_2H_2P_2O_7$. Yield: 11 g. Found: Ag 60·2%, P 14·1%. Calc. for mixture: Ag 60·1%, P 14·1%.

Preparation of an acid silver pyrophosphate of the approximate formula $Ag_3H_5P_4O_{14}$. 10 g. of silver pyrophosphate ($Ag_4P_2O_7$; prepared by precipitation of $Na_4P_2O_7$ with $AgNO_3$) were mixed with 60 g. pyrophosphoric acid and heated in an oil bath of 150° for 1 hr. After cooling the mass was dissolved in about 200 ml. acetone. An insoluble residue was filtered off. The acetone extract was poured into 2000 ml. acetone, the white precipitate was allowed to settle and was finally centrifuged, washed with acetone and ether and dried. Found: Ag 47·98%, P 18·12%. Calc. for $Ag_3H_5P_4O_{14}$: Ag 47·90%, P 18·35%.

Preparation of monosodium pyrophosphate. 2 g. $Na_4P_2O_7$, anhydrous, were mixed with 8 ml. pyrophosphoric acid and gently heated in a test tube over a Bunsen flame until dissolved. After cooling, the mass was dissolved in 600 ml. acetone and 200 ml. ether were added. An oily precipitate formed which was allowed to settle and was hardened by treatment with fresh acetone. The precipitate was centrifuged and dried *in vacuo* on a porous plate. It had a glassy appearance and was very hygroscopic. Found: Na (by uranyl zinc precipitation): 10·11%, P 27·28%. Na : P = 1·0 : 2·0. Calc. for $NaH_3P_2O_7$: Na 11·49%, P 31·01%.

Preparation of bromo-aneurin. 1 g. aneurin hydrochloride was dissolved in 60 ml. anhydrous acetic acid at about 80°. The solution was saturated with dry gaseous HBr with careful exclusion of moisture. The temperature was first kept at about 50° to prevent the aneurin from crystallizing out; it was gradually lowered to 0° and the saturation was completed at this temperature. Sometimes a turbidity appeared owing to the precipitation of aneurin hydrobromide, but this is of no importance. The solution was now heated in sealed tubes for 3 hr. at 130°. After cooling, the combined liquids were precipitated with 300 ml. ether; the precipitate was centrifuged, washed with ether and dried *in vacuo* over moist KOH until all HBr had been absorbed. It was now dissolved in 20–25 ml. of hot methyl alcohol. On cooling in ice the solution usually remained clear; if any unchanged aneurin was present, it crystallized out at this stage. The solution was warmed again and 5 ml. ether were added. On cooling, the bulk of the bromo-aneurin crystallized out in long felted needles. The substance is sufficiently pure for the preparation of cocarboxylase. For analysis it was twice more crystallized from methanol-ether. Yield: mean of 7 preparations: 754 mg. Highest yield: 1·00 g., lowest yield: 565 mg. M.P. 234° (decomposition). Calc. for $C_{12}H_{17}N_4SBr_3$: ionizable Br 32·69%, total Br 49·03%, C 29·45%, H 3·51%, N 11·46%. Found: ionizable Br 32·60%; 32·78%. Total Br (method of Robertson [1915]) 49·13%. Analysis of C, H and N (Weiler): C 29·13%, H 3·71%, N 11·0%.

Preparation of cocarboxylase from bromo-aneurin. 1·2 g. $Ag_2H_2P_2O_7$ were mixed with 5 ml. pyrophosphoric acid and heated in an oil bath at 150° until dissolved. The mixture was allowed to cool to about 80°. 600 mg. bromo-aneurin were now added and heated in an oil bath at 100° for 40 min. with constant stirring. After cooling, the mass was ground up with 90 ml. acetone and centrifuged. The precipitate was washed with 2 more lots of 90 ml. acetone. It was now treated with 50 ml. ice-cold water, the insoluble residue was centrifuged and washed once with 20 ml. water and once with 10 ml. $N/10$ HNO_3. The combined extracts were brought to *p*H about 6 with cold saturated $Ba(OH)_2$ (about 35 ml.) and Ag^+ was removed with H_2S. The precipitate was centrifuged

off and well washed with water 3 times. The solution was concentrated *in vacuo* at 30° to about 60 ml. After cooling in ice, cold saturated $Ba(OH)_2$ (about 10 ml.) was added until the reaction was alkaline to thymolphthalein. The precipitate was quickly centrifuged off and the supernatant fluid was tested for the appearance of a further precipitate on addition of $Ba(OH)_2$. If it remained clear it was immediately neutralized with N H_2SO_4 (1·5–2 ml.) and again centrifuged. Both precipitates were well washed.

At this stage the solution was usually made up to 100 ml. and aliquots were analysed for total organic and acid-hydrolysable P and for catalytic activity. Also the insoluble residue of AgBr was reprecipitated and weighed. The following is the result of a typical experiment: AgBr after dissolving in conc. NH_3 and reprecipitation with HNO_3: 688·4 mg. Calc. for 600 mg. bromo-aneurin: 690 mg. Found: total organic P 47·5 mg., acid-hydrolysable P 11·8 mg. Acid-hydrolysable P : total P = 1 : 4·03. 11·8 mg. acid-hydrolysable P indicate 183 mg. cocarboxylase which account for 187 mg. bromo-aneurin. 47·5 mg. $- 2 \times 11{\cdot}8$ mg. $= 23{\cdot}9$ mg. P indicate 321 mg. monophosphate which account for 376 mg. bromo-aneurin. Amount of bromo-aneurin phosphorylated: 563 mg. = 94 %. Amount of cocarboxylase present calculated from the assay of catalytic activity by comparison with a pure sample of synthetic cocarboxylase: between 180 and 190 mg.

The isolation was continued as follows: the solution which had been made up to 100 ml. was acidified by the addition of 8 ml. 10 N H_2SO_4. After removal of the precipitated $BaSO_4$ the solution was precipitated with 10 ml. 25 % phosphotungstic acid. The precipitate was centrifuged and washed with 0·8 N HCl. It was now dissolved in 40 ml. of a mixture of 1 part N/10 HCl and 2 parts acetone, an insoluble residue was centrifuged off and 130 ml. acetone were added. After standing overnight the supernatant solution was set aside for the preparation of the monophosphate and the gummy precipitate was washed with acetone, dissolved in 2 ml. N/10 HCl and precipitated with a mixture of 4 ml. alcohol and 16 ml. acetone. The following day the precipitate was dissolved in 30 ml. water and 5 ml. N $AgNO_3$ were added. The precipitate of AgCl was removed and washed with N/100 HNO_3. The solution was adjusted to *p*H 7 with N/10 NH_3. The precipitate of the silver salt was decomposed with H_2S and the solution freed from H_2S *in vacuo*. 5 N HCl was added to give a concentration of 0·8 N. The phosphotungstic acid precipitation was repeated with 10 ml. of a 25 % solution and the precipitate was dissolved in 9 ml. of the HCl-acetone mixture. The clear solution was precipitated by the addition of 30 ml. acetone. The crystalline precipitate was once more recrystallized from alcohol-acetone, as above, and finally dissolved in 1 ml. N/10 HCl and precipitated with 4 ml. alcohol. Yield: 107 mg. After two more recrystallizations from dilute alcohol the following analyses were obtained: Total organic P: 12·92 %; acid-hydrolysable P 6·50 %. Analyses of C, H and N (Weiler): C 30·42 %, H 4·52 %, N 11·5 %. Calc. for cocarboxylase: C 30·08, H 4·42, N 11·71, total P 12·93, acid-hydrolysable P 6·45 %.

From the mother liquors of the first acetone precipitation the monophosphate was precipitated in fine needles by the addition of 150 ml. acetone. The precipitate was repeatedly recrystallized by dissolving in a few ml. of N/10 HCl and adding acetone, acetone-alcohol and finally alcohol. Yield: 180 mg. M.P. 199°. Total P 7·55 % (calc. 7·45 %). No-acid hydrolysable P.

Preparation of cocarboxylase and aneurin monophosphate by the method of Weijlard & Tauber. 1 g. aneurin was used as the starting material. The procedure of Weijlard & Tauber was strictly adhered to up to the $Ba(OH)_2$ pre-

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cipitation and the subsequent removal of Ba^{++} with H_2SO_4. The solution was then made up to 100 ml. and analysed. It contained 120·0 mg. of total P and 29·9 mg. of acid-hydrolysable P. Acid hydrolysable P : total P = 1 : 4·0. From these figures the formation of

460 mg. cocarboxylase, accounting for	349 mg. aneurin
and of 807 mg. monophosphate, accounting for	688 mg. aneurin
	1037 mg. aneurin

was calculated, i.e. a phosphorylation of 100 %. However, the enzymic assay of the catalytic activity revealed the presence of only 220–230 mg. cocarboxylase, i.e. about half of what was to be expected from the determination of the acid-hydrolysable P.

The isolation of the two esters proceeded on the lines described in the preceding section. Cocarboxylase: 118 mg. Total P 13·70 %, acid-hydrolysable P 6·26 %. Monophosphate: 260 mg. Total P 8·0 %, acid-hydrolysable P 0.

Attempt to prepare aneurin phosphates from bromo-aneurin and monosodium pyrophosphate: 500 mg. $Na_4P_2O_7$ (anhydrous) were dissolved in 2 ml. pyrophosphoric acid by gentle heating and 250 mg. bromo-aneurin were added. The mixture was heated in an oil bath of 120° for 2 hr. and mechanically stirred. Found in the solution after removal of inorganic P.

Acid-hydrolysable P 0·15 mg. ~ 2·32 mg. cocarboxylase	~ 2·37 mg. bromo-aneurin
Total P 0·60 mg. ~ 4·03 mg. monophosphate	~ 4·72 mg. bromo-aneurin
	7·09 mg. bromo-aneurin

Acid-hydrolysable P : total P = 1 : 4·0. 2·84 % phosphorylation.

Titration of the second phosphoric ester of aneurin before and after heating with acid. Titrations were carried out with exclusion of atmospheric CO_2 with CO_2 free, 0·0196 *N* NaOH against phenolphthalein. 36·75 mg. aneurin hydrochloride ~ 5·28 ml. NaOH (1·00 mol.). 32·50 mg. of the product isolated from the mother liquors of the cocarboxylase fraction (7·55 % P) ~ 8·00 ml. NaOH (2·01 mol.). 37·44 mg. isolated from a solution of the above substance which had been heated with *N* H_2SO_4 for 2 hr. ~ 9·20 ml. NaOH (2·01 mol.).

Precipitation of aneurin phosphates by salts of heavy metals. (1) With mercuric acetate: 5 ml. 1·3 % mercuric acetate solution were added to 10 ml. of a solution of aneurin phosphates containing 0·75 mg. of acid-hydrolysable P and 2·94 mg. of total P. NaOH was added to *p*H 7. Supernatant contained 0·024 mg. of acid-hydrolysable P and 0·815 mg. of total P. Precipitate contained 0·73 mg. of acid-hydrolysable P and 2·10 mg. of total P.

(2) With uranyl acetate: 2 ml. 10 % uranyl acetate solution were added to 10 ml. of the same solution of aneurin phosphates. Neutralized with NaOH. Supernatant: acid-hydrolysable P 0·02 mg. Total P 0·068 mg. Precipitate: acid-hydrolysable P 0·73 mg. Total P 2·83 mg. Similar results were obtained with bismuth nitrate, cuprous chloride, lead acetate and vanadium chloride.

Appendix

The reaction of aneurin and aneurin phosphates with nitrous acid

Most authors agree that aneurin reacts only very slowly, if at all, with nitrous acid. According to Williams & Spies [1938] the reaction takes place only in the presence of mineral acid with heat.

I have studied the reaction by the method of Warburg *et al.* [1935]. The manometer vessels described by Dickens & Šimer [1930] for the measurement of the R.Q. in phosphate medium were found very suitable. 0·1 ml. of the solution

containing the amino compound was accurately delivered into the circular groove together with 0·1 ml. glacial acetic acid. The side bulb contained 0·4 ml. 35 % barium nitrite solution and the central compartment 2 ml. alkaline $KMnO_4$. The gas space was filled with oxygen-free N_2. The reaction was carried out at 20°. After a short time for temperature equilibration the taps were closed, a reading was taken and the barium nitrite solution was tipped into the circular groove. The manometers were left without shaking for the desired time. At the end of the reaction the alkaline $KMnO_4$ was mixed with the other solutions.

Table 2. *Reactions of aneurin, aneurin monophosphate and aneurin pyrophosphate with nitrous acid*

Addition	Time allowed for reaction hr.	N_2 evolved µl.	% of theory
4·33 mg. aneurin	3	184	68
3·61 mg. cocarboxylase	3	15	8·9
3·16 mg. aneurin	5	164	82·5
2·83 mg. aneurin	6	139	78
3·68 mg. aneurin monophosphate	6	28·5	14·4
2·90 mg. cocarboxylase	6	39	28·8

Table 2 shows that aneurin does react with nitrous acid though very slowly. It has not been possible to obtain a theoretical yield of N_2. But it is noteworthy that the amino group is still less reactive in the phosphate esters.

A mechanism of enzymic decarboxylation has recently been proposed [Weil-Malherbe, 1940] in which the first step is the formation of a Schiff base as originally suggested by Langenbeck [1933]. Stern & Melnick [1939] have independently discussed a similar scheme, but they seem to think that the low reactivity of the NH_2 group makes it improbable that such a reaction occurs. But obviously the properties of the NH_2 group may be profoundly altered in combination with the enzyme.

Summary

1. A method is described for the synthesis of cocarboxylase by the interaction of "bromo-aneurin" and silver pyrophosphate.

2. Although no water is liberated during the reaction, it is found that the ratio of acid-hydrolysable P : total P = 1 : 4. This is interpreted as meaning that for each molecule of the primary pyrophosphoric ester (cocarboxylase) another molecule of the secondary symmetrical pyrophosphoric ester is formed. The same ratio of acid-hydrolysable P : total P is found if the phosphorylation of aneurin is carried out by the method of Weijlard & Tauber, or by the interaction of "bromo-aneurin" and monosodium pyrophosphate.

3. The separation of the primary and secondary pyrophosphoric esters is possible owing to the greater solubility of the phosphotungstate (or the reineckate) of the secondary ester in aqueous acetone. A modified procedure of purification is described.

4. The secondary pyrophosphoric ester seems to be a very unstable and easily hydrolysable compound, since the substance isolated is aneurin monophosphate, as shown by titration.

5. The catalytic activities of various samples of crystalline cocarboxylase prepared either by the new method or by the method of Weijlard & Tauber are identical, but are only 60 % of the activity of natural cocarboxylase.

6. Aneurin phosphates are precipitated in neutral solution by the ions of several heavy metals.

7. The reactions of aneurin and aneurin phosphates with nitrous acid were investigated. After 5 hr. about 80 % of the theoretical amount of N_2 was evolved from aneurin, but much less from the phosphoric esters.

I am greatly indebted to Dr F. Bergel of Roche Products Ltd. whose generous supplies of aneurin made this work possible and to Mr J. Weijlard for a sample of cocarboxylase. I also wish to acknowledge the help given by Dr W. Kelly, especially during the experiments leading up to the preparation of bromoaneurin, and by Mr L. A. Stocken of the Oxford Laboratory, who carried out the comparison of the catalytic activities of synthetic and natural cocarboxylase.

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118. THE ZINC AND COPPER CONTENTS OF THE ORGANS AND TISSUES OF CHINESE SUBJECTS

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(*Received 15 March 1940*)

It is now a recognized fact that the two metals Zn and Cu are with a few doubtful exceptions universally present in biological material. The published data show some disagreement regarding the actual amounts to be expected. For example, Scott & Fisher's [1938] value for the Zn content of pancreas is 10 times as great as values reported by Lutz [1926] and by Hattori [1929], and the values for the Cu content of various organs reported by White (quoted by Herkel [1930]) and by Chou & Adolph [1935] are considerably higher than those reported by Herkel [1930], Cunningham [1931] and Tompsett [1934]. The results reported by some authors have been based on analyses of only a few samples, while the analytical technique employed is in some instances open to question.

Comparison with the amount of a metal in the blood will show whether or not it is concentrated in an organ or tissue, while comparison of the relative amounts of two metals present in an organ and in blood will show if either is preferentially retained. This last-mentioned point of difference may help to reveal a special property or function.

It is not known whether biological relationships exist between Cu and Zn although it is known that both form compounds with proteins. Only Bodansky [1921] has determined both metals in the same material.

Methods

The post-mortem material used was in no case more than 24 hr. old. In at least 12 cases samples were obtained within 6 hr. of death. After transportation to the laboratory in sealed jars to prevent water loss the specimens were prepared for analysis by removing adherent fat and connective tissue and cutting them into small pieces. A representative sample was dried for analysis and the remainder weighed and dried to constant weight. Some workers have adopted the technique of washing the organ or tissue free from blood regardless of the possibility that other soluble materials may be removed.

The "dithizone" method of Sylvester & Hughes [1936] was used for isolating the two metals from the ashed material. The Zn was determined iodimetrically by the method of Lang [1929; 1933] and the Cu colorimetrically with diethyldithiocarbamate after McFarlane [1932]. Minor modifications have been described elsewhere by Eggleton [1940].

Provided that a temperature of 450° is not exceeded, dry ashing does not result in loss of Zn or Cu. Wet ashing involves the use of relatively large quantities of acid and alkali which cannot be freed sufficiently from traces of Zn and Cu which are always present, and analyses cannot be performed accurately on small quantities of material owing to the disproportionately high blanks.

The oedematous condition of several cases under examination made it necessary always to express results in terms of dry matter. Greater uniformity could be obtained by expressing the results in terms of fat-free dry matter since

fat also can serve as a diluent in some tissues. However, this was not considered sufficient justification for the extra labour involved in what was at this stage a general rather than a detailed investigation.

Results

The blood of patients under treatment for beriberi, nutritional oedema and general malnutrition, whose pre-hospital diets are characterized by a small amount of protein and a larger amount of carbohydrate, has been shown by Eggleton [1940] to contain less Zn and somewhat more Cu than the blood of individuals whose diet approximates to normal. The relevant values obtained are summarized below for reference.

Table 1. *Zn and Cu contents of blood, mg./kg. dry matter*

	No. of cases	Zn	Cu	Zn/Cu
Foreign (well nourished)	4	30·5	7·3	4·2
Chinese (well nourished)	4	32·3	8·1	4·0
Chinese (ill nourished)	55	18·0	9·9	1·82

In continuation of this study it was primarily desired to compare the Zn and Cu contents of the organs and tissues of subjects coming within these two dietary categories. The pre-hospital dietary history of beriberi subjects can be inferred, that of other patients is often difficult to assess with accuracy, whereas that of accident cases can be guessed only from the appearance of the body at death. These difficulties together with the existence of secondary complications in most cases made it impossible to obtain a satisfactory comparison. For this reason the original intention was abandoned and data were collected from all cases that came to the autopsy table.

The Zn and Cu status of human organs and tissues

The figures for Zn here reported agree reasonably well with those reported by Rost & Weitzel [1919] for brain, liver and muscle, by Lutz [1926] for brain, liver, kidney and testicle and by Hattori [1929] for brain, liver, kidney, pancreas and heart. Figures for liver published by Raoult & Breton [1877], van Itallie & von Eck [1913], Herkel [1930] and Lundegaardh [1934] are also in agreement. The figures of Cristol [1923] for liver and spleen and of Bertrand & Vladescos [1913] for testicle are relatively high whereas those of Hattori [1929] for spleen are relatively low. The value found by Simakov [1936] for pectoral muscle is also relatively low.

Where comparisons are possible the figures for Cu agree satisfactorily with those reported by Bodansky [1921] for brain, by Herkel [1930] for liver, kidney, pancreas, spleen and thyroid, by Cunningham [1931] for brain, liver, kidney, pancreas and spleen and by Tompsett [1934] for brain, liver and kidney, spleen and pancreas. The figures reported by Chou & Adolph [1935] for liver, kidney, pancreas, spleen, heart, lung and muscle are all considerably higher, as are the figures given by White (quoted by Herkel [1930]) for liver, kidney, pancreas, spleen and heart.

The mean values obtained are in good agreement with those published by the majority of other workers, which, in view of the varied nature of the cases under consideration, is of some significance, for it suggests that "normal" mean values can be derived approximately from a series of hospital cases chosen without

Table 2. *Distribution of Zn and Cu in the organs and tissues of Chinese subjects, mg./kg. dry matter*

Sex and age	Cerebrum		Cerebellum		Liver		Kidney		Suprarenal		Pancreas		Spleen		Heart		Lung		Muscle	
	Zn	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn	Cu
F 04	55*	12·0*	60*	8·2*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F 9	47*	14·2*	54	27·9	116	31·2	218	7·4	—	—	216	6·3	74	5·9	79	10·7	76	5·3	—	—
M 13	—	—	—	—	—	—	—	—	—	—	121	—	—	—	—	—	—	—	—	—
F 16	—	—	—	—	—	—	—	—	—	—	116*	12·4	—	—	—	—	—	—	—	—
F 17	33*	18·1*	45*	37·8*	186*	33·3*	122	—	—	—	—	—	61*	5·6	88*	18·0	—	—	—	—
M 18	46*	16·0*	60*	21·8*	545	48·1	245	15·0	106	6·8	135	4·6	72	8·5	80	11·3	149	10·0	307	7·3
F 19	—	—	—	—	178	32·4	68	11·6	—	15·3	169	14·2	80*	13·1*	118	18·6	—	—	—	—
F 25†	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	206	4·0
M 26	—	—	—	—	347	32·7	257	30·1	89	8·6	215	7·6	68	3·5	113	11·2	43	8·3	121*	7·7*
M 28	41	19·3	58	27·1	260	21·7	248	8·2	76	14·3	158	11·0	71	8·9	136	20·4	63	13·5	283	9·2
M 30	47*	18·9*	62*	29·3*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
M 31	44*	15·7*	54*	26·2*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
M 32	—	—	—	—	562	93·4	260	12·3	97	9·0	146	11·5	66	8·0	86*	8·1	62	12·1	181*	6·5
M 32*a*	38	19·3	55	25·3	285	68·6	157	8·3	106*	26·7*	—	—	79	13·3*	—	—	71	8·2	—	—
M 33	53	—	69	—	123	—	137	—	—	—	—	—	—	—	66	—	—	—	—	—
M 34	51*	18·2*	54*	25·4*	164*	53·2*	160*	14·1*	—	—	—	—	87*	8·1*	139*	12·7*	—	—	—	—
M 35	44*	21·0*	56*	38·2*	172*	26·7*	179*	46·1*	—	—	115*	7·8	76*	5·9*	87*	15·3*	—	—	—	—
M 38	55*	24·7*	55*	35·9*	169	25·5*	89*	11·6*	—	—	44	9·7	85	8·1*	86	18·2*	—	—	—	—
M 40	34*	23·0	42*	48·8	183*	99·9*	155*	13·9*	—	—	78	—	69*	4·7*	84*	13·4	—	—	—	—
M 43	42*	18·1*	51*	22·3*	190*	15·3*	209	7·6	72*	6·8*	128	4·1	62	4·2	92	9·4	57	6·5	267	5·1
M 45	32*	17·0*	51*	26·8*	226*	34·9*	314*	18·7	—	—	93	—	73*	6·4*	93*	17·0	—	—	—	—
M 48	43	—	53	—	162	—	146	—	—	—	—	—	—	—	126	—	—	—	—	—
M 51	40*	17·2*	46	22·6*	308	47·1	239	9·2	40	11·2	160	8·8	64	9·4	—	—	—	—	—	—
M 58†	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	164	8·2
F 60	—	—	—	—	154	18·4	149	10·0	72	5·4	139	8·2	62	4·8	105*	8·7	49	10·9	234	4·4
M ?	39	17·9*	60	34·9	399	23·9	238	4·6	98	10·0	132	7·0	70	5·7	118	13·7	43	7·0	—	—
? ?	32	17·0	53	30·4	166	17·1	129	10·2	64	4·1	—	—	73	5·8	112*	7·8	60	7·0	271	5·2
Mean Zn and Cu	43	18·1	55	28·8	245	40·2	186	14·1	82	10·7	135	8·7	72	7·2	100	13·4	67	8·9	226	6·4
Mean ash % in d.m.	6·13		6·93		4·13		4·27		3·24		4·62		5·03		3·99		4·64		3·76	
Mean ratio Zn:Cu	2·38		1·91		6·1		13·2		7·7		15·5		10·0		7·5		7·5		35·3	

The thyroids of M 32*a*, F 60, M ? and ? ? contained respectively, 116, 168, 105* and 71 p.p.m. Zn and 24·6 7·1, 3·3* and 5·9 p.p.m. Cu. The thymus of M 13 contained 121 p.p.m. Zn and that of M 40 49 p.p.m. Zn. The testicles of M 18 and M 32*a* contained 54 and 69 p.p.m. Zn and 6·3 and 19·4 p.p.m. Cu respectively. The dura mater of M 33 contained 56·8* p.p.m. Zn and 7·7* p.p.m. Cu; the choroid plexus and sciatic nerve contained respectively 57·1 and 45·5 p.p.m. of Zn. The dura of M 35 contained 18·4 and 4·3 p.p.m. of Zn and Cu respectively and the gall bladder and bile fluid contained 31·3 p.p.m. and 14·6 of Zn respectively. The choroid plexus and sciatic nerve of M 48 contained 99·6 and 24·3 p.p.m. of Zn respectively.

* Figures marked with an asterisk have been confirmed by duplicate analyses at least.

† Sample taken from living subject at amputation.

special regard to pathological condition. It is probable that the observed variability arises more from differences in dietary habit than from pathological conditions, except in so far as chronic disease may influence the quantity or quality of food ingested.

Cerebrum and cerebellum. In the present investigation 17 brains were analysed for the Zn and Cu contents of both cerebrum and cerebellum. The results show that cerebellum contains more Zn and Cu than cerebrum, that both contain on the average less Zn than any other organ or tissue examined, that both, but especially cerebellum, contain larger amounts of Cu than any other organ or tissue examined with the single exception of liver and that the Zn and Cu contents of brain shows only a small range of variation. The amount of Zn in the dry matter of brain is less than twice the amount of Zn in the dry matter of blood, whereas the amount of Cu is 3–4 times as great; the ratio Zn/Cu is much smaller than in any other organ or tissue, even liver. From these facts it is concluded that Cu is selectively concentrated by these organs, particularly by the cerebellum, though the reason is not understood.

Suprarenal, spleen, lung, thymus and testicle. These all contain, in terms of dry matter, about twice as much Zn as blood, but the amount of Cu in these organs is of the same order as in blood. Zn is therefore selectively concentrated to a similar extent in all these organs.

Heart muscle and voluntary muscle. There is a marked difference between voluntary muscle and involuntary muscle in respect of Cu and Zn contents. In voluntary muscle (*Pectoralis major* and *Rectus abdominalis*) the mean Zn content is high (226)—as high as in hair, teeth, liver and kidney. The mean Cu content is low (6·4) and is not significantly different from the amount in blood. In heart muscle the mean amount of Zn (100) is less than half that present in voluntary muscle but the mean Cu content (13·4) is more than twice as much. Whereas only Zn is concentrated in voluntary muscle both metals are concentrated in heart muscle.

Liver. Although usually greater in liver the amounts of Zn and Cu are more variable than in any other organ or tissue examined. A high Cu content does not necessarily accompany a high Zn content or *vice versa*. However the ratio Zn/Cu in liver is on the average approximately the same as in blood, which indicates that both metals are in general concentrated in unchanged proportions.

Kidney. This organ also shows considerable variability in Zn and Cu contents. Although both metals are concentrated the ratio Zn/Cu is twice that of blood, indicating that Zn is concentrated in the kidney to a greater extent than Cu.

Pancreas. The Zn content of this organ is occasionally comparable with that of liver and kidney. The amount of Cu, however, is seldom greater than that in blood. Zn therefore is selectively concentrated.

Thyroid. The four results obtained suggest that Zn is normally concentrated in the thyroid, whereas Cu is not.

Influence of nutrition on Zn and Cu contents

Of special interest (Table 3) is the high Zn content, and possibly also high Cu content, of the liver and kidney in those cases which were obviously ill nourished. This is taken to indicate that under starvation conditions these two organs are so depleted of fat, glycogen and other matter with which these metals are not normally associated, that the concentration is increased in terms of residual dry matter. This effect is more noticeable with Zn than with Cu and in the liver than in the kidney. It is probable that the proteins with which the Zn and Cu occur in combination may be regarded as being always in a state of saturation in

Table 3. *Zn and Cu contents of liver and kidney in well and ill nourished subjects*

Well or fairly well nourished patients	Ash % d.m.	Zn p.p.m.d.m.	Zn % in ash	Cu p.p.m.d.m.	Cu % in ash	Ratio Zn/Cu
			Liver			
F 17	3·64	186	0·51	33·3	0·092	5·6
M 34	3·41	164	0·48	53·2	0·156	3·1
M 38	4·42	169	0·38	25·5	0·058	6·6
F 60	3·79	154	0·41	18·4	0·049	8·4
F 19	4·07	178	0·44	32·4	0·080	5·5
M 28†	4·93	260	0·53	21·7	0·044	12·0
Mean	4·04	185	0·46	30·8	0·080	6·9
Ill nourished patients						
M ?	5·04	399	0·79	25·9	0·051	15·4
M 26	3·53	347	0·98	32·7	0·093	10·6
M 51	4·33	308	0·71	47·1	0·109	6·5
M 32	4·66	562	1·21	93·4	0·200	6·0
M 18	4·62	545	1·18	48·1	0·104	11·3
Mean	4·44	432	0·97	49·4	0·112	10·0
Well or fairly well nourished patients			Kidney			
F 17	4·59	122	0·27	—	—	—
M 34	3·71	160	0·43	14·1	0·038	11·4
M 38	3·57	89*	0·25	11·7	0·033	7·6
F 60	3·67	149	0·41	10·1	0·028	14·8
F 19	3·39	68*	0·20	11·6	0·034	5·9
M 28†	4·11	267	0·54	8·2	0·020	30·0
Mean	3·84	143	0·35	11·1	0·031	13·9
Ill nourished patients						
M ?	4·79	238	0·50	4·6	0·010	51·7
M 26	3·36	257	0·77	30·1	0·090	8·5
M 51	3·62	239	0·66	9·2	0·025	26·0
M 32	4·03	260	0·65	12·3	0·031	21·1
M 18	5·32	245	0·46	15·0	0·028	16·3
Mean	4·22	248	0·61	14·2	0·037	24·8

* Nephritis.

† In spite of the clinician's description it would appear that M 28 should be regarded as poorly nourished.

respect of these metals, so that the amount found is in this sense an inverse measure of the degree of fatty infiltration conditioned by the diet. Although analyses were not made it would appear likely on this basis that the Zn content of an organ is positively correlated with its N content. It is not possible to make suitably accurate comparisons with other organs and tissues owing to paucity of data but the indications are that they are affected only slightly in this respect.

Discussion

It is frequently stated that certain organs "accumulate" metals, the implication being that the organ has an affinity for the metal which is accumulated for a specific purpose. Foetal liver is said to accumulate Zn and Cu, an argument advanced in support of the belief that these metals are indispensable to life.

This is no argument in itself, for the higher concentration may be due to the fact that the amount of some major constituent of the liver normally present

and of which these metals are independent, is deposited in the liver to an increased extent after birth, thereby lowering their concentration in terms of dry matter. If metals are associated in organic combination with proteins then the other constituents with which they are not associated will act as diluents to varying degrees.

Eggleton [1940] has reported the lowered Zn content and slightly increased Cu content of the blood in acute, subacute and chronic beriberi and in cases of protein deficiency and nutritional oedema. It was thought that this condition might be reflected in the Zn and Cu contents of the organs. However, this is not so. The explanation would appear to be that, whereas a low Zn intake such as occurs in malnutrition involving low protein, produces a low Zn level in the blood, the liver and kidney are depleted of fats, cholesterol, glycogen etc. to a relatively greater extent in the aggregate than of protein under these conditions, so that the concentration of Zn and Cu is increased in terms of residual dry matter. However, it is still not understood why blood Cu should be increased while blood Zn is decreased.

It is considered that little information is to be gained by studying the changes in concentration of isolated metallic constituents of organs in various pathological or nutritional states unless they are considered in conjunction with changes in other relatively more bulky constituents. Nevertheless, the figures here reported have some value, for it is necessary to know the highest concentration of a metal which may be reached under all conditions except where the metal in question has been ingested in unnatural quantities. For this reason it is important to consider the amount present relative to the total ash content or to the amount of some other metal known to be a regular constituent.

The results show that whereas the Zn contents of all tissues studied are in general higher than that of the perfusing blood, the Cu contents are higher only in liver, brain and possibly also in kidney and heart. Why other organs should not have their proportionate share of Cu is not clear, unless it be that there is some important difference in respect of the compounds with which these metals can unite.

Summary

An investigation into the distribution of Zn and Cu in the human body, involving over 300 Zn and 200 Cu analyses of 13 different organs and tissues from 26 Chinese subjects of both sexes, ranging in age from 156 days to 60 years and dying from various causes, has shown that both metals are always present. Comparison with the average amounts of these metals present in the dry matter of blood shows that Zn is concentrated in all organs and tissues examined, but that Cu is concentrated only in liver, brain, kidney and heart in order of descending magnitude.

Cerebrum and cerebellum contain less Zn and, with the exception of liver, more Cu than any organ or tissue examined. Cerebellum contains more Zn and Cu than cerebrum.

The increased concentration of Zn and Cu in the liver and kidney observed in cases of malnutrition is thought to be due to the relatively greater withdrawal of the fats and carbohydrates from these organs, than of the proteins with which the metals remain in association.

I am indebted to Dr P. B. Wilkinson of the Medical Dept., Hongkong, to Dr C. L. Dale of St Luke's No. 2 Hospital, Shanghai and to my colleague Dr L. S. Kau for supply of autopsy material and for access to their records.

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119. THE EFFECT OF THE LEVEL OF CALCIUM INTAKE ON THE BIOLOGICAL VALUE OF A PROTEIN

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(*Received 29 May 1940*)

Ranganathan & Rau [1938] have reported that the addition of 2% $CaCO_3$ to a typical Indian diet, the "Madras diet" containing 0·124% Ca, increased the biological value of the protein, as measured on rats when fed at a 10% level of intake, from 44 to 76. In view of the importance and wide implications of this claim it was decided to submit it to independent test. If confirmed it would certainly provide a further argument in favour of increased use of milk in vegetarian dietaries based mainly on cereals.

To make differences in Ca intake more pronounced a "synthetic" diet was used in our experiments. A protein of high biological value, heat-coagulated egg white, was employed to facilitate the detection of any depressing effect of Ca deficiency.

During the course of our experiments Swaminathan [1939] published results which showed that the level of Ca intake had no effect on the biological value of the proteins of a "typical poor South Indian diet". Our results are in complete agreement with his findings.

Experimental

The biological value of the protein was determined by the method of Mitchell [Mitchell, 1924; Mitchell & Carman, 1926]. Six pairs of litter-mate female rats weighing 49–58 g. were placed in metabolic cages when 25 days old. The cages, vitamin additions, methods of collection and of analysis of the excreta and the low egg-nitrogen diet used in the standardizing period were as described in detail by Henry *et al.* [1937]. The composition and analyses of the "low" and

Table 1. *Composition and analysis of the experimental diets*

	"High" Ca	"Low" Ca
Butter-fat	15·25	15·38
Rice starch	45·76	46·14
Potato starch	8·97	9·05
Castor sugar	15·25	15·38
Ca- and P-free salts*	2·62	2·64
$CaHPO_4, 2H_2O$	1·87	—
$NaH_2PO_4, 2H_2O$	—	1·71
$CaCO_3$	0·72	0·12
Egg albumin†	9·56	9·56
% N	1·352	1·294
% Ca	0·8600	0·0694
% P‡	0·4306	0·3699
% Moisture	8·54	9·06

* Henry & Kon [1937].

† Commercial dried egg white was dissolved in water, coagulated by heating to 195° F. for 7½ min. and dried again.

‡ The disagreement in the P content of the diets was due to an error in the calculation of the amount of $NaH_2PO_4, 2H_2O$ to be added to the "low Ca" diet.

"high" Ca diets are given in Table 1. The diets contained approximately 8·5% protein (N × 6·25). The "low" Ca diet contained 0·07%, the "high" 0·86% Ca. Litter-mates were allocated by lot to the two diets and the intake was equalized within pairs. Both groups received their respective diets for two consecutive complete metabolic periods (20 days in all) to emphasize, if possible, any effects of low Ca feeding. At the end of the experiment the animals were killed with coal gas, the humeri and femora were removed and their ash contents were determined.

Results

The results of the experiment are shown in Tables 2–5. Table 2 gives the individual figures for the nitrogen metabolism data; Table 3 gives mean figures for the biological values and true digestibilities. Table 4 gives data on the Ca intakes of the animals and the bone ash contents of their bones. Table 5 shows the statistical treatment of differences. The statistical analyses were made by the paired *t*-test of "Student" [1908; 1925] as described by White [1937].

Table 2. *Nitrogen metabolism; individual data for 6-day periods*

Rat no.	Level of Ca intake	Initial wt. g.	Final wt. g.	Diet intake g.	N intake mg.	Faecal N mg.	N in urine mg.	Biological value	True digestibility
				Low egg-nitrogen diet					
1	—	59	65	46·23	—	88·6	80·2	—	—
2	—	56	61	44·89	—	83·6	82·8	—	—
3	—	55	62	51·00	—	101·9	82·4	—	—
4	—	57	65	51·00	—	92·9	85·0	—	—
5	—	53	56	44·47	—	90·8	83·2	—	—
6	—	49	53	42·64	—	81·7	79·8	—	—
7	—	51	56	40·91	—	86·5	73·7	—	—
8	—	50	56	43·96	—	*	78·7	—	—
9	—	52	57	39·45	—	75·8	75·4	—	—
10	—	52	54	39·50	—	77·5	79·1	—	—
11	—	53	58	48·62	—	111·9	82·4	—	—
12	—	54	61	49·00	—	95·1	77·6	—	—
				1st experimental period					
1	High	76	86	44·00	594·7	102·8	140·8	92·8	98·7
2	Low	70	83	43·74	566·0	74·3	118·7	96·3	100·0
3	High	71	93	51·48	695·9	93·9	128·0	96·1	100·0
4	Low	74	95	51·48	666·2	69·3	124·5	97·4	100·0
5	High	66	74	38·83	524·9	89·2	129·9	93·3	98·4
6	Low	62	73	37·59	486·4	83·8	135·7	91·7	97·7
7	High	66	70	34·31	463·8	101·6	130·1	91·1	94·9
8	Low	63	77	40·50	522·8	75·8	134·3	92·0	100·0
9	High	64	71	38·18	516·1	129·1	217·3	73·3	89·9
10	Low	67	78	43·50	562·9	61·4	139·3	93·1	100·0
11	High	69	87	55·00	743·4	125·4	128·8	96·6	100·0
12	Low	72	90	55·00	711·7	81·6	145·6	94·1	100·0
				2nd experimental period					
1	High	92	109	58·61	792·2	137·5	183·5	91·3	100·0
2	Low	91	109	59·38	768·4	112·6	176·4	91·1	100·0
3	High	102	120	61·69	833·9	129·9	194·1	90·7	100·0
4	Low	103	116	58·76	760·4	97·4	210·3	88·6	100·0
5	High	81	98	56·45	763·0	127·7	155·8	93·9	98·9
6	Low	80	97	55·85	722·7	110·6	181·2	90·9	97·4
7	High	75	85	48·62	657·2	123·4	210·6	83·7	99·1
8	Low	80	96	51·50	666·4	104·6	179·7	91·0	99·9
9	High	72	78	40·41	546·2	116·6	203·7	80·5	94·3
10	Low	84	91	42·50	550·0	56·6	199·0	84·1	100·0
11	High	95	107	51·88	701·3	131·4	168·0	93·1	98·7
12	Low	100	107	53·50	692·3	80·5	304·2	74·4	100·0

Table 2 (*cont.*)

Rat no.	Level of Ca intake	Initial wt. g.	Final wt. g.	Diet intake g.	N intake mg.	Faecal N mg.	N in urine mg.	Biological value	True digestibility
				Low egg-nitrogen diet					
1	—	118	122	57·50	—	151·6	126·7	—	—
2	—	113	115	51·36	—	133·8	114·9	—	—
3	—	122	127	49·24	—	131·5	111·3	—	—
4	—	118	124	56·00	—	113·8	120·3	—	—
5	—	105	115	63·50	—	137·2	117·8	—	—
6	—	103	115	62·79	—	124·3	129·3	—	—
7	—	90	92	35·46	—	91·0	116·6	—	—
8	—	95	98	46·50	—	94·4	125·7	—	—
9	—	83	90	42·70	—	94·1	119·6	—	—
10	—	94	99	46·50	—	113·5	111·7	—	—
11	—	109	117	58·50	—	139·4	118·9	—	—
12	—	111	120	58·50	—	120·0	135·1	—	—

* Analysis lost. For the calculation of the basal metabolic faecal N of this rat the mean value obtained for the remaining rats was used.

Table 3. *Mean biological values and true digestibilities of egg-white in low or high Ca diets*

	High Ca intake		Low Ca intake	
	Biological value	True digestibility	Biological value	True digestibility
Period I	90·52	96·98	94·10	99·62
Period II	88·88	98·50	86·68	99·55
Mean	89·70	97·74	90·39	99·58

Table 4. *Gains in weight, Ca intakes and ash content of dry, defatted femora and humeri of rats receiving the high and low Ca diets*

		1st experimental period			2nd experimental period			
Rat no.	Level of Ca intake	Gain in wt. g.	Ca intake mg.	Ca, mg. intake/g. gain in wt.	Gain in wt. g.	Ca intake mg.	Ca, mg. intake/g. gain in wt.	% bone ash*
1	High	10	378·4	37·84	17	504·0	29·65	59·9
2	Low	13	30·4	2·34	18	41·2	2·29	55·8
3	High	22	442·7	20·12	18	530·5	29·47	60·0
4	Low	21	35·7	1·70	13	40·8	3·14	56·4
5	High	8	333·9	41·74	17	485·5	28·56	58·6
6	Low	11	26·1	2·37	17	38·8	2·28	55·3
7	High	4	295·1	73·78	10	418·1	41·81	57·4
8	Low	14	28·1	2·01	16	35·7	2·23	56·6
9	High	7	328·3	46·90	6	347·5	57·92	57·5
10	Low	11	30·2	2·75	7	29·5	4·21	57·5
11	High	18	473·0	26·28	12	446·2	37·18	59·1
12	Low	18	38·2	2·12	7	37·1	5·30	57·6
Mean High Ca intake		11·5	375·2	41·11	13·3	455·3	37·43	58·75
Mean Low Ca intake		14·7	31·5	2·22	13·0	37·2	3·24	56·53

* Each figure is the mean of duplicate determinations on one femur and one humerus.

It can be seen from Table 3 that the biological values of the proteins of egg-white were very similar at both levels of Ca intake. In the first experimental period a higher value was given by the "low" Ca group while in the second period the "high" Ca group was slightly superior. Table 5 shows that the differences lack any statistical significance either for the separate or the pooled periods. True digestibilities were in both periods higher in the "low" Ca group but the differences between the two groups were again statistically not significant.

Table 5. *Statistical treatment of the results*

		Difference high-low Ca	Standard error of mean	P*
Biological value	Period I	−3·58	±3·37	1 : 1
	Period II	+2·20	±3·67	1 : 1
	Periods I and II	−0·69	±2·52	1 : 1
True digestibility	Period I	−2·64	±1·72	1 : 5
	Period II	−1·05	±1·06	1 : 3
	Periods I and II	−1·84	±0·98	1 : 12
% bone ash		+2·22	±0·68	1 : 44*a*

* P = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population. *a* = over 1 : 20.

The percentages of bone ash in both groups of animals were 58·7 and 56·5 for the "high" and "low" Ca diets respectively. At first sight the figure of 56·5 % for animals receiving only 2–3 mg. Ca/g. gain in weight would seem high; it must be remembered, however, that the experimental periods (totalling 20 days) were sandwiched between two periods (10 days each) in which the supply of Ca was adequate. Table 5 shows that the % bone ash of the "high" Ca rats was significantly greater than that of the "low" Ca animals.

According to Sherman & Macleod [1925] female rats growing from 60 to 110 g. (the range covered in this experiment) normally deposit about 10 mg. Ca/g. gain in weight. It can be seen from Table 4 that the intake for the "high" Ca group was considerably above this figure (41 and 37 mg./g. gain respectively for the 1st and 2nd periods) while the figure of 2·2–3·2 mg. for the "low" Ca group was much below the normal. The difference in Ca intakes was probably sufficiently marked for any fall in the biological value of protein caused by low Ca intake to become evident. As no differences were found it must be concluded that the level of Ca intake had, in these experiments, no effect on the biological value or digestibility of the protein. This is in complete agreement with the findings of Swaminathan [1939] but in opposition to the report of Ranganathan & Rau [1938].

Summary

1. The biological values of the nitrogen of two diets, both containing egg white as a source of protein but one having a Ca content of 0·86 % and the other of 0·07 %, were measured on rats by the balance sheet method of Mitchell.

2. No differences were found in the biological values and true digestibilities of the protein of the two diets. The availability of dietary nitrogen is apparently not decreased when the diet is deficient in Ca.

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120. THE RELATIONSHIP OF CARBOHYDRATE METABOLISM TO PROTEIN METABOLISM

2. NOTE ON THE EFFECT OF SEPARATION IN TIME OF THE PROTEIN AND CARBOHYDRATE MOIETIES OF THE DIET OF THE ADULT AND GROWING RAT

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(*Received 20 May 1940*)

Previous observations on the adult human subject have shown that when the protein and carbohydrate moieties of an adequate diet are separately ingested over short periods of time, fat being associated with both moieties, a negative N and S balance develops [Cuthbertson & Munro, 1939]. The following experiments were designed to determine if this phenomenon also took place in the case of the adult rat and, further, if the growth of young rats would thereby be affected. This is an extension of a previous brief note [Cuthbertson *et al.* 1939].

Experimental

The animals were housed in zinc cages and the arrangement for urine collection was that of Hopkins. All urines were made up to constant volume before their total N content was determined by the macro-Kjeldahl method. To prevent loss of NH_3 the filter funnels beneath the cages were washed down with dilute H_2SO_4 every morning before feeding and there was always some acid present in the collecting vessels.

Adult rats

Exp. 1. 4 adult male rats of some 300 g. body weight were placed on a mixed diet of the following composition for 1 week:

Dried egg yolk	12 %
Dried egg albumin	6 %
Potato starch (uncooked)	17 %
Rice starch (uncooked)	40 %
Cane sugar	10 %
Butter	10 %
De Loureiro's salt mixture [1931]	5 %
Brewer's yeast extract (untreated 0·01 % acetic acid extract of Chick & Roscoe [1930])	2 ml. daily supplied in separate dish
Cod liver oil	2 drops daily

The mean daily intake per rat during the week was 17·5 g.

During the next 12 days the diet was separated into two fractions:

A		B	
Potato starch	17 parts	Dried egg yolk	12 parts
Rice starch	40 ,,	Dried egg albumin	6 ,,
Cane sugar	10 ,,	Butter	5 ,,
Butter	5 ,,	Salt mixture	2·5 ,,
Salt mixture	2·5 ,,		
Cod liver oil	2 drops daily		
Yeast extract	2 ml. daily		

The A fraction (carbohydrate+fat) was fed *ad lib.* at 8.30 a.m. and the uneaten residue removed and weighed at 12 noon. 4·0 g. of the B fraction (protein+fat) were fed at 4.30 p.m. to each animal and were always consumed within a few hours. After 5 days on this diet the animals were weighed (fasting) and then there followed a 7-day period of urine collection on the same dietary regime.

At the end of a week the animals were reweighed (fasting) when it was found that all had lost weight, the mean loss per rat during this period (period 1) being 7 g. The rats were then given the A and B fractions in a mixed form (period 2), the amount consumed daily being the mean value of the daily intakes during period 1 for the animal concerned. During period 2 there was a gain in weight by all the animals, the mean gain per rat being 5 g.

The mean daily output of urinary N was greater in all 4 animals during the period of separation of their dietary protein and carbohydrate (0·165 g. N) than during the period of mixed feeding (0·145 g. N). The individual differences in the levels of N excretion were 0·0182, 0·0151, 0·0200 and 0·0266 g. per day (mean 0·02 g.).

Exp. 2. The foregoing experiment was repeated with a batch of 8 mature male rats of some 300 g. weight: margarine was substituted for butter. On an average they consumed 18 g. of the mixed diet during the pre-separation period. Thereafter they were subjected to a period of separation (period 1), 4·5 g. of the B moiety being fed per day. On the 5th day of separation urine collection was commenced and continued for a fortnight, when the A and B moieties were fed mixed for a further week (period 2), in quantities corresponding to the mean of the two preceding weeks.

During the 1st week of urine collection the average loss of weight was 6 g. per rat and by the end of the 2nd week they had lost a further 3 g. The mean daily output of urinary N for the 1st week of urinary collection during separation was 0·185 g. per rat and 0·200 g. during the 2nd week. This difference was probably due to the smaller intake of the carbohydrate+fat moiety (A fraction) during the 2nd week, 5·91 g. per rat as compared with 6·58 g. during the 1st week. This loss of appetite for A fraction of the diet is a constant feature in such separation experiments, but the B fraction was in all cases consumed ravenously.

During the week of mixed feeding the average gain in body weight was 5 g. per rat. Two of the animals, however, showed a further fall, one slight the other of 10 g. The mean urinary N output during this period was 0·180 g. per day, 0·0125 g. below the mean of the two preceding weeks of separation. This difference is undoubtedly less marked than that observed in Exp. 1, namely 0·02 g. This may have been due either to the more prolonged period of separation, with its progressive loss of appetite inducing a state from which recovery is slow, or to a gradual readjustment by the animal to the changed dietary conditions. 7 of the 8 animals showed N retention during period 2, the mean daily values being

0·0501, 0·0036, 0·0154, 0·0054, 0·0050, 0·0132 and 0·0140 g. The animal which had exhibited a further and considerable reduction in weight during this period also showed a diminished N retention.

It is evident from these two experiments that the separation of the A and B fractions of the diet, essentially a separation of the time of ingestion of the protein and carbohydrate moieties, leads to a loss in weight and tissue N. In this respect these observations on the adult rat agree with those on adult man [Cuthbertson & Munro, 1939].

Young rats

Two paired feeding experiments were carried through. In the first the diet was similar to that already described (Exp. 1) protein being associated with some of the fat+salt mixture. In the second the diet was slightly different and the protein component was fed alone.

Exp. 3. 12 pairs of recently weaned male litter-mates were fed the mixed diet *ad lib.* for a period of 12 days. One member of each of the first 10 pairs was then selected for the separated diet and was fed 5 g. of the B moiety at 8·30 a.m., the uneaten residue being taken away at 12 noon. The A moiety (fed *ad lib.*) was given to these rats ("X" rats) at 4.30 p.m. and taken away at 10 p.m. The second member of each pair (control or "Y" rats) then received the exact amount of food eaten the previous day by its opposite, but in a mixed form. There was thus always a lag of 1 day in the quota of food supplied to the controls. The two remaining pairs ("Z" rats) were fed the mixed diet *ad lib.* throughout the whole growth period.

In the earlier stages of the experiment, when the controls had not finished their food by 12 noon the dish was taken away and replaced at 4.30 p.m. In all instances the controls ate all the food provided. With increasing weight the intake of the B fraction which averaged 2·5 g. initially, rose to 5 g./day. It was kept stationary at that level for a further 10 days and was then reduced to 2·5 g. daily as the higher protein intake appeared to be accelerating the growth of these animals to a greater extent than was occurring in the Z group fed the mixed diet *ad lib.* By reducing the B fraction to 2·5 g. daily it was possible to bring the protein intake more into line with this 3rd group. The mean intake per rat per day at the end of the experiment was 2·5 g. B+7·27 g. A.

The animals in this and the experiment to follow were weighed in the fasting state. In the 2nd experiment the precaution was taken of reserving until 4.30 p.m. a quota of the total food due to the controls. This was carried out on the days preceding the weighing of the rats, i.e. every 4th day.

Growth data. The total period of separation of the A and B components of the diet was 42 days. During this period the mean increase in body weight per rat was 106 g. (mean initial weight 66 g.), that of the controls fed the same quota of food but mixed, 114 g. (mean initial weight 69 g.). Those animals fed the mixed diet to full satisfaction showed an increase of 130 g. (mean initial weight 69 g.). The growth curves have not been included as no real difference existed between the "separated animals" and the "controls" fed the same amount of food, but mixed.

Exp. 4. The two moieties, C and D, were constituted as shown on the next page.

The mixed diet was fed to 12 pairs of recently weaned male litter-mates. 2 pairs continued on the mixed diet and were fed *ad lib.*, the remaining 10 pairs after being on the mixed diet for 10 days were separated and a paired feeding

experiment was carried through which was similar to the preceding one except that moiety D, was fed in the late afternoon and C in the morning.

C (Fed in excess at 9 a.m. Residue removed at 12 noon)		D (Fed at 4.30 p.m. Residue, if any, removed at 10 p.m.)	
Potato starch (uncooked)	17 parts	Ashless casein (Glaxo A/E)	12 parts
Rice starch (uncooked)	40 ,,	Dried egg albumin	6 ,,
Cane sugar	10 ,,		
Lard	10 ,,		
Salt mixture	5 ,,		
Cod liver oil	3 drops daily per rat		
Yeast extract	2 ml. daily per rat		

The urinary N excretion of 4 pairs of these rats was determined. The collection of urine was from the 2nd to the 10th day of the period of separation, and for the controls fed the same quota of food but in a mixed form, from the 3rd to the 11th day.

The D moiety never exceeded 1·5 g. throughout the whole growth experiment. In order to minimize any possible differences in weight which might have been attributable to a difference in the time of feeding, 1·5 g. of the total quota of food due to the controls were reserved until 4.30 p.m. on the day preceding the actual weighing.

N balance data. The mean daily urinary N excretion per rat fed the separated diet (mean body weight 87 g.) was 0·0928 g., that of the controls receiving the same quota of food but in a mixed form was 0·0930 g. (mean body weight 87 g.).

In contrast to the adult rat the young rat exhibits no significant differences in N excretion when placed on a diet in which the protein element is separated from the carbohydrate.

Growth data. The period of separation of the C and D fractions of the diet lasted 32 days. During this period the mean increase in body weight of the rats receiving the separated diet was 32 g. per rat, that of the controls fed the same quota of food but mixed, 32·5 g. Those animals fed the mixed diet to full satisfaction exhibited a mean increase in weight of 67 g.

The differences in the rates of growth observable in these two growth experiments is probably primarily due to the qualitative and quantitative differences in protein intake. The second group had a more restricted protein intake and qualitatively the proteins may have been slightly inferior.

Discussion

The growth behaviour of these young rats indicates that the separation in the time of ingestion of the protein and carbohydrate moieties of the diet leads to a loss of appetite which is not subsequently overcome. This loss of appetite is related to the carbohydrate plus fat fraction and not to the protein fraction which is always eaten voraciously. In contradistinction to the adult rat and adult human subject the metabolism of protein in the young rat remains unaffected. In the adult organism there is a loss of body N which although of small degree and very close in amount to the minimum endogenous expenditure, is almost always present.

The experiments on the adult organism have admittedly been of relatively short duration as compared with the growth experiments and it might be considered that, given time, the organism would adapt itself to the abnormal conditions. We have, however, noted that over like periods of time during the early days of the "separation" period, this difference in behaviour does exist between the young and mature rat. In the second experiment on the adult rats it was thought that some evidence on the time factor might be obtained by observing the difference in N excretion during the first and second weeks of urine collection during "separation". Undoubtedly there was a greater loss of N during the second week but this was complicated by the fact that the animals, although eating the same amount of protein plus fat, had definitely reduced their intake of carbohydrate plus fat. It is difficult to see how this experimental difficulty can be overcome.

The resiliency of the growing organism must be such as to lead to a readjustment to the abnormal dietary conditions so as to maintain development as nearly as possible at the optimal level permitted by the gross intake. How does this come about and why cannot the adult organism similarly respond? Undoubtedly the endocrine system acts as a regulator of metabolic phenomena. Does the lack of dietary carbohydrate during a considerable part of the day lead to a defective secretion of insulin with consequent defective oxidation of carbohydrate and increased protein metabolism? Young [1940] considers that the growth-promoting action of the anterior lobe in the young animal may possibly rest on the ability of the pancreas to secrete sufficient insulin to induce N retention and promote carbohydrate oxidation. Mirsky & Swadesh [1938] believe that the anterior lobe of the pituitary exerts two types of action on protein metabolism in the normal animal: (1) direct stimulation of protein catabolism in the muscles; (2) anabolic, by way of its effect on the pancreas. During growth the influence of the pituitary would appear then to be contingent on its pancreatropic activity. There is the evidence of Anselmino *et al.* [1933] and Richardson & Young [1937] that treatment of rats with anterior lobe extracts increases the islet tissue in the rat. More recently Marks & Young [1940] have shown that the insulin content of rat pancreas is greatly increased under the same conditions. In view of this evidence experiments have now been commenced to determine if injections of growth-promoting extracts of the anterior pituitary can induce retention of N during periods when the carbohydrate and protein elements in the diet are being separately ingested.

In view of our observations on the adult human organism it might be asked how the Eskimo metabolizes his protein. The observations of Rabinowitch [1936] and many others go to show that the Eskimo does eat carbohydrate. For 2 months in the year berries, lichens etc., are available and throughout the rest of the year he obtains his supplies from the glycogen present in the liver, muscle and skin of animals. He relishes the stomach contents of the caribou and musk ox and the clams found in the walrus stomach.

The evidence of Mirsky *et al.* [1937] indicates that in the eviscerated dog, which is essentially a muscle preparation, the spontaneous output of amino-acid N is removed under the stimulus of insulin and the amino-acids are presumably utilized for storage by the muscle or for synthesis into peptides or proteins. It may be that the restitution of the endogenous N expenditure is dependent on some such action by insulin and that in the absence of a stimulus this cannot be effected. In the adult the necessary stimulus is dietary carbohydrate ingested along with protein; in the young the anterior pituitary creates the necessary stimulus to insulin production even in the absence of the dietary stimulus.

From the observations on mature animals it would appear that the association of fat with protein does not affect the loss of N during the separation in time of the ingestion of protein and carbohydrate. This is of particular interest with reference to the problems of the interconversion of these three proximate principles. The stimulus of dietary carbohydrate is presumably the missing factor. The minute amounts of carbohydrate normally associated with proteins are not sufficient in themselves to effect this.

In the light of these and our earlier findings [Cuthbertson & Munro, 1937; 1939][1] we are led to the conclusion that there is an intimate relationship between the metabolism of carbohydrate and protein and that the time factor and also the relative and absolute quantities of these two components in the diet as well as the frequency of feeding can effect the metabolism of protein. Fat undoubtedly also plays a part but this is more obscure and is chiefly related to the relative and absolute amounts associated with carbohydrate. Lovell & Rabinowitch [1939] have confirmed that time is a factor which influences the protein-sparing action of carbohydrates in the treatment of diabetics on low calorie diets.

SUMMARY

Separation in the time of ingestion of the protein and carbohydrate moieties of an otherwise adequate diet leads in the case of the young rat to a loss of appetite but to no apparent disability to metabolize protein. On the other hand in the mature rat such a procedure is coupled with a small loss of nitrogen and body weight. In respect of this behaviour the mature rat corresponds to the adult human subject.

We wish to express our thanks to Prof. E. P. Cathcart and to Prof. G. M. Wishart for their helpful criticism. We are also indebted to the Rankin Medical Research Fund and to the D. C. Andrew Fund for grants in aid of this research.

[1] In Table 5 of this paper [Cuthbertson & Munro, 1939], for calories derived from fat in period II read 748 for 493 and 776 for 521.

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121. ON THE MECHANISM OF FERMENTATION OF TREHALOSE BY *FUSARIUM LINI* BOLLEY

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(*Received 27 May 1940*)

It has been pointed out and experimentally proved by several authors [Krebs, 1935; Hofstetter *et al.* 1938; Macfarlane, 1939; Deuticke & Hollmann, 1939; Shaw & Barker, 1939] that the phase sequence of the enzymic conversions of different substrates is dependent on the actual state of the cells harbouring the enzymes concerned. These authors imply that there exists virtually no proof for the common assumption that evidence of the course of degradations or dissimilations of this kind, obtained with the aid of artificial preparations, represents the actual course of the phase sequence within the living cell.

Recent attempts to determine the actual phase sequence during the alcoholic fermentation of carbohydrates appear especially informative in this respect. In a recent review [Nord, 1940] of progress in regard to the mechanism of alcoholic fermentation by yeasts, fusaria, *Zymosarcina ventriculi* and *Thermobacterium mobile* Lindner, it is pointed out that not only do certain living fusaria possess the capacity to convert carbohydrates enzymically by three independent phase sequences, but also that ordinary living yeasts do not follow exclusively one path in the course of sugar dissimilation.

This does not necessarily mean that results obtained with an enzyme preparation have to be regarded as contradictory to the course of the reaction within the living cell. It suggests, however, the suitability of tracing the mechanism of degradation of certain compounds by using enzymically comparable living organisms where the existing data, obtained with the aid of artificial enzymic preparations, do not clarify the actual phase sequence within the original living system. A search of the literature [Armstrong & Armstrong, 1934; Myrbäck *et al.* 1937; Myrbäck, 1939; Nord & Engel, 1938; Dammann, 1938] revealed the following facts. First, maltose and lactose may be fermented by living *Fusarium lini* Bolley directly as well as indirectly, and are not, in the case of direct fermentation, necessarily phosphorylated. No observable phosphorylation accompanies the onset of fermentation of these disaccharides. Second, living *F. lini* Bolley contains an enzyme which enables it to ferment dextrin with the evolution of CO_2 and the formation of ethyl alcohol. Third, no decisive data whatsoever are available concerning the question of fermentability and the mechanism of fermentation of trehalose by yeasts.

At the suggestion of Mr Nord, I have undertaken a series of experiments to decide upon the fermentability of added, not native, trehalose by means of living *F. lini* Bolley.

Experimental

The following nutrient medium was used for all experiments:
100 ml. distilled water.
1·29 g. KH_2PO_4 (approx. 300 mg. P per 100 ml.).
0·08 g. $MgSO_4, 7H_2O$.
0·02 g. KNO_3.
Carbohydrate in amounts designated in each experiment.

The initial *p*H of the medium was 4·4. The water content of crystalline trehalose was taken into consideration in calculation of amounts of trehalose used. All inoculations were made with mycelium from agar cultures of *F. lini* Bolley. CO_2 was determined according to the method of Truog [1915] with a modification whereby ascarite was employed for removal of CO_2 before aeration of flasks.

In preliminary experiments, measurement of CO_2 indicated that added trehalose ferments at a rate comparable with that of glucose (cf. Table 1).

In the series of experiments in Table 2, the carbohydrate concentration was 1%. The first measurements were made on the second day after inoculation. It is to be observed that the CO_2 evolution in the case of trehalose fermentation reaches a higher maximum than in glucose fermentation.

Table 1

	mg. CO_2 evolved daily from 0·75% solutions of	
Day	Glucose	Trehalose
1	—	—
2	14·6	27·6
3	44·6	51·5
4	55·5	61·4
5	92·8	139·9
6	62·3	118·0
7	26·5	82·0

Table 2

	Daily CO_2 evolution in mg.			
	Glucose		Trehalose	
Day	I	II	III	IV
1	—	—	—	—
2	14·2	12·6	19·9	19·6
3	61·3	56·0	45·9	26·9
4	88·2	116·6	90·7	100·4
5	102·3	112·0	112·9	123·3
6	76·4	95·5	112·4	134·7
7	72·0	83·1	145·2	140·5
8	—	54·6	82·3	—
10	43·5	37·7	60·5	44·2
11	19·7	20·2	19·0	12·2
15	5·6	5·0	5·9	4·3

During the course of the fermentation process, the *p*H of the solutions changed to different degrees, the trehalose solutions more nearly maintaining the initial value of 4·4. After three weeks, the *p*H of the glucose solutions was 4·9 while the trehalose solutions showed a value of 4·6.

In the case of experiments where trehalose was the carbon source, growth was observed 6 to 8 hr. in advance of any perceptible development of mycelium in the glucose cultures. After three weeks, the mycelium from flasks used for CO_2 determinations was separated from nutrient medium, rinsed and dried in an oven for 1 hr. at 80° on filter paper which had been previously dried and weighed. The weights show very clearly the preferential growth of *F. lini* Bolley on trehalose. The dry weights of mycelium from two trehalose cultures were 120 and 138 mg., while those from two glucose cultures were 82 and 86 mg. The media contained 100 ml. of 1 % carbohydrate solution.

The course of the dissimilation of trehalose and of glucose was followed polarimetrically. Solutions containing 1 g. of carbohydrate per 30 ml. were used in these experiments in order to give readings sufficiently large to permit detection of slight differences. Samples for this determination were removed by means of a sterile pipette, filtered rapidly and transferred without dilution to polarimeter tubes. Results of such a series of experiments are shown in Table 3. The specific rotations for crystalline trehalose and for glucose are +178·3° and +52·6° respectively.

Table 3

	Polarimeter readings				Sugar content calculated in g. per 30 ml.			
	Trehalose		Glucose		Trehalose		Glucose	
Day	I	II	III	IV	I	II	III	IV
0	+6·394°	+6·388°	+1·765°	+1·698°	1·08	1·08	1·00	0·97
1	6·426	6·462	1·684	1·698	1·08	1·08	0·96	0·97
2	6·498	6·308	1·636	1·666	1·09	1·06	0·93	0·95
3	6·128	6·075	1·515	1·505	1·03	1·02	0·86	0·86
4	5·394	5·456	1·466	1·472	0·91	0·92	0·83	0·84
5	4·512	4·546	1·418	1·456	0·76	0·76	0·81	0·83
6	3·43	3·45	1·398	1·45	0·58	0·58	0·80	0·73
7	2·04	2·05	—	—	0·34	0·34	—	—
8	0·79	—	1·21	—	0·15	—	0·69	—
9	0·12	0·125	1·08	1·11	0·002	0·002	0·62	0·63
15	0·00	0·00	1·00	1·01	0·00	0·00	0·57	0·57

It is significant that the rate of disappearance of trehalose exceeds the rate of glucose dissimilation. Likewise the rate of disappearance of trehalose conforms closely to the rate of CO_2 evolution in trehalose fermentation (cf. Fig. 1).

In several series of experiments undertaken with the idea of determining whether trehalose can be phosphorylated by means of added inorganic P, or to compare the speed of esterification of trehalose with that of the phosphorylation of glucose at an initial *p*H of 4·4, it was found that trehalose is esterified practically at the same rate and to the same extent as glucose (3–4 % on the 6th day). Phosphate was determined according to Fiske & Subbarow [1925]. It appears, however, to be questionable whether this observation, in connexion with the optical measurements of the rate of disappearance of trehalose in the course of its dissimilation, can be reconciled, at least at present, with the considerations of Lipmann [1935] in regard to the glycolysis of polysaccharides with the aid of rabbit muscle extracts.

Discussion and conclusions

Unlike living yeasts, living *F. lini* Bolley ferments trehalose more rapidly than glucose. Growth of the organism is more rapid and more abundant on trehalose than on glucose. Since the rate of trehalose dissimilation is more rapid

than that of glucose the occurrence of the latter as an intermediary is excluded, for, in such a case, the dissimilation rate of glucose would be the controlling step. The indications thus far observed point toward a *direct* fermentation of this disaccharide. Glucose has never been observed in a fermenting trehalose mixture and, consequently, we cannot say that under our experimental conditions there was a trehalase active or present in *F. lini* Bolley.

The culture of *F. lini* Bolley No. 5140 was received from the Biologische Reichanstalt in Berlin-Dahlem through the courtesy of Dr Wollenweber. The trehalose for our experiments was obtained from two sources. Pure crystalline

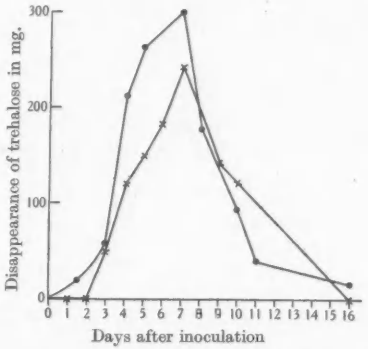

Fig. 1. Comparison of optical activity and CO_2 evolution in the course of trehalose fermentation.
×—× Measured according to optical activity.
•—• Measured according to CO_2 evolution.

trehalose was supplied through the assistance of Dr Gudernatsch, Nutley, N.J., by Messrs Hofmann-La Roche and Co. Ltd. in Berlin. A pure product was also isolated from dried top yeast, furnished through the kindness of Dr C. N. Frey of Fleischmann Laboratories, New York.

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122. A MICRO-DETERMINATION OF SUCCINIC ACID

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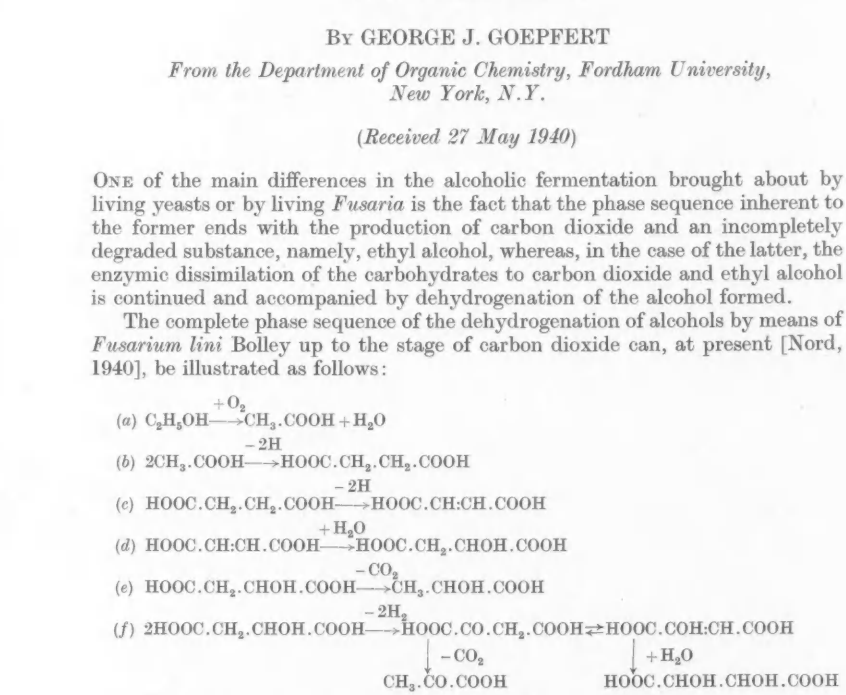
(*Received 27 May 1940*)

One of the main differences in the alcoholic fermentation brought about by living yeasts or by living *Fusaria* is the fact that the phase sequence inherent to the former ends with the production of carbon dioxide and an incompletely degraded substance, namely, ethyl alcohol, whereas, in the case of the latter, the enzymic dissimilation of the carbohydrates to carbon dioxide and ethyl alcohol is continued and accompanied by dehydrogenation of the alcohol formed.

The complete phase sequence of the dehydrogenation of alcohols by means of *Fusarium lini* Bolley up to the stage of carbon dioxide can, at present [Nord, 1940], be illustrated as follows:


(a) $C_2H_5OH \xrightarrow{+O_2} CH_3.COOH + H_2O$
(b) $2CH_3.COOH \xrightarrow{-2H} HOOC.CH_2.CH_2.COOH$
(c) $HOOC.CH_2.CH_2.COOH \xrightarrow{-2H} HOOC.CH{:}CH.COOH$
(d) $HOOC.CH{:}CH.COOH \xrightarrow{+H_2O} HOOC.CH_2.CHOH.COOH$
(e) $HOOC.CH_2.CHOH.COOH \xrightarrow{-CO_2} CH_3.CHOH.COOH$
(f) $2HOOC.CH_2.CHOH.COOH \xrightarrow{-2H_2} HOOC.CO.CH_2.COOH \rightleftarrows HOOC.COH{:}CH.COOH$
$HOOC.CO.CH_2.COOH \xrightarrow{-CO_2} CH_3.CO.COOH$
$HOOC.COH{:}CH.COOH \xrightarrow{+H_2O} HOOC.CHOH.CHOH.COOH$

With the exception of fumaric acid and oxaloacetic acid, all the compounds appearing in this scheme, in which malic acid possesses a central position following acetic and succinic acids, were experimentally demonstrated. However, no actual mechanism is so far known for the transient formation of one of the most important C_4 compounds, viz. succinic acid, from acetic acid, which contrary to earlier convictions [Meyerhof, 1936] can not only be accumulated but also isolated without the introduction of fixatives, etc. [Rotini *et al.* 1936].

A rapid and accurate method for the routine determination of succinic acid was therefore deemed necessary, all the more since (*a*) the method of Denigès [1936] used in earlier work for qualitative detection could not be developed as a quantitative one and (*b*) the procedures suggested, e.g. by Rau [1893], Bordas *et al.* [1898], Moyle [1924], Clutterbuck [1929] and Bernhauer [1939], were limited in application.

I have therefore undertaken, at the suggestion of Mr Nord, the determination of minute quantities of succinic acid in nutrient media and in the presence of other organic acids.

Experimental

1. *Removal of volatile acids and acids oxidized by potassium permanganate. Extraction of succinic acid*

The solution containing 2 ml. 0·1 *N* succinic acid, volatile acids and nutrient media containing NO_3^-, $SO_4^=$ and $PO_4^{\equiv}$, was acidified with conc. H_2SO_4 until acid to Congo red. It was then heated on the water bath and treated with 0·1 *N* $KMnO_4$ until a brown precipitate was obtained. This was then dissolved by addition of Na_2SO_3 and the solution evaporated to dryness. The residue was dissolved in 15 ml. of chloride-free distilled water; 2 ml. conc. H_2SO_4 were added and the solution was saturated with K_2SO_4. The whole was transferred quantitatively to an extractor [McNair, 1933] and the casserole washed with three successive portions of saturated K_2SO_4 (4 ml., 3 ml., 3 ml.).

After extracting for 3–4 hr. with ether, the latter was transferred to a flask and 5 ml. of chloride-free distilled water added. The ether was removed by distillation on a water bath and the aqueous residue boiled over an open flame for 30 sec. The flask was then cooled under the tap and one drop of *m*-nitrophenol indicator added. The analysis was then carried out as in the control titration. The recovery determined so far on 25 samples amounts to $98 \pm 2\%$.

2. *Control titration*

2–4 ml. of 0·1 *N* succinic acid were placed in a flask and diluted to 5 ml. with chloride-free distilled water or a known weight of succinic acid was dissolved in 5 ml. of chloride-free distilled water. As an indicator one drop of *m*-nitrophenol (0·3 % in water) was used; 0·05 *N* NaOH was added until one drop caused the solution to turn yellow. It was decolored by means of one drop of 0·1 *N* HNO_3 and a measured excess of 0·02 *M* $AgNO_3$ added immediately. The solution was brought back to neutral by the addition of one drop of 0·05 *N* NH_4OH. The precipitate was kept for 1·5–2 hr. in the dark. The solution was filtered through a Gooch crucible or a fritted glass filter and washed quantitatively from the flask on to the filter by three successive portions of 1 % NH_4NO_3 (3 ml., 3 ml., 2 ml.). Two drops of dichlorofluorescein indicator (0·1 % in 70 % ethanol) and 7–9 drops of 1 % soluble starch (chloride-free) were added to the filtrate. The excess silver was then titrated with 0·02 *M* KBr until the pink colour disappeared. One ml. of 0·02 *M* $AgNO_3$ is equivalent to 1·18 mg. succinic acid. Recovery based on 40 samples was $99{\cdot}5 \pm 0{\cdot}5\%$.

Summary

1. An accurate and rapid method is presented for the estimation of quantities of succinic acid as low as 10 mg. This method has the advantage that it can be carried out without difficulty with the usual laboratory equipment.

2. The reproducibility of the actual titration has been established by using *m*-nitrophenol and dichlorofluorescein as indicators along with potassium bromide.

3. The time of extraction of succinic acid from the solution has been decreased to 3–4 hr.

To the Rev. Dr F. W. Power, I wish to express my thanks for helpful suggestions.

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123. THE NITROGEN CYCLE IN BIOLOGICAL SYSTEMS

1. SOME CONDITIONS AFFECTING THE DISTRIBUTION OF NITROGENOUS COMPOUNDS DURING TREATMENT OF SEWAGE BY THE ACTIVATED SLUDGE PROCESS

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(*Received 29 April 1940*)

In methods of treatment of sewage, the object is usually to reduce the amount of readily oxidizable matter, so that the treated effluent does not cause serious depletion of the dissolved O_2 content of a river or stream into which it is discharged. In the activated sludge process, after preliminary screening and sedimentation, the sewage is vigorously aerated for several hours in admixture with activated sludge. The mixture withdrawn from the aeration tanks is allowed to settle, the treated effluent is discharged, and a proportion of the settled activated sludge is returned to the aeration tanks and used again. Usually the process is continuous and the period of aeration is controlled by adjusting the rate of flow of the sewage.

Activated sludge is prepared by aeration of sewage and consists largely of bacterial cells, some alive, some dead [Wooldridge & Standfast, 1933], together with smaller amounts of granular material. Though it is known that nitrification of the ammoniacal N in sewage usually occurs during aeration with activated sludge, and that nitrite is frequently detected as an intermediate compound, there is little precise knowledge of the conditions which determine the course of the N cycle in the process under consideration. The object of the present investigation was to obtain a clearer understanding of this problem.

Of the N in the settled sewage employed in the experimental work described in this paper, usually about 90% was present as ammonia-N,[1] the balance occurring in organic form: the N in the sludge was present almost entirely in organic form.

Vigorous aeration of sewage with biologically active sludge results in a more or less rapid disappearance of the ammonia-N from the liquid phase. Under usual conditions, part of this ammonia-N is converted into organic form and becomes incorporated in the sludge, while the balance is oxidized to nitrite and then to nitrate. Examination of published results from activated sludge plants in English towns has shown that the fraction of the ammonia-N in the crude sewage which is subsequently found as nitrite or nitrate in the treated effluent varies within very wide limits; sometimes there is no oxidized N in the final effluent.

[1] Except where otherwise stated, no distinction is made here between ammonia- and ammonium-N.

Experimental technique

The settled sewage used in these experiments was supplied from the Finchley Sewage Disposal Works, through the kindness of the Borough Engineer. The activated sludge was made in the laboratory and was maintained in an active condition by continuous aeration and daily additions of sewage. Usually, mixtures for experimental work were taken within a period of 5–60 min. after the addition of fresh sewage to the stock of activated sludge.

In the main, the experiments were concerned with the changes in the nitrogenous compounds during aeration of mixtures of sewage and activated sludge, either alone or after addition of N compounds.

In the first experiments, bubbles of air were passed through 2-l. mixtures of crude sewage and activated sludge, to which nitrogenous compounds were sometimes added, in Winchester bottles at laboratory temperature: the proportion of activated sludge was between 10 and 20% by volume (Tables 1, 2, 4–7). Samples of 100 ml. of the mixture were withdrawn at intervals; the liquid was separated from the sludge by centrifuging, and the two phases were analysed separately. In experiments of not more than 3 days' duration, no account was taken of the slight losses of water due to evaporation since these necessitated a correction of less than 1% in the amount of total N.

In further experiments, mixtures of sewage and sludge containing different amounts of added nitrogenous compounds were aerated continuously with ammonia-free air for several days in 500 ml. flasks at laboratory temperature (Tables 3, 8). The distribution of the nitrogenous compounds was determined at the beginning and end of the experiments. Any ammonia liberated from the mixtures and removed in the current of air was collected and estimated. The liquid and sludge were separated by filtration and were analysed separately.

Adsorption of ammonium ions by activated sludge

Some experiments were carried out in which 2·5 l. of a mixture of settled sewage and activated sludge were aerated for periods of 24 hr. Samples of 100 ml. of the mixture were withdrawn periodically and, after centrifuging for 5 min. to remove suspended matter, the concentrations of ammonia- and of nitrite- and nitrate-N in the liquid were determined. The adsorbed ammonium-N in the sludge was determined by the method of Olsen [1929], in which the sludge, after having been thoroughly washed with water, was added to 50 ml. *N* KCl, the *p*H value was adjusted to 1·0 with HCl and the mixture was made up to 200 ml. with distilled water. After shaking for 1 hr., the mixture was filtered and the ammonia-N in the filtrate was determined (Table 1). The results, which

Table 1. *Adsorption of ammonium ions by activated sludge during aeration of mixtures of sewage and activated sludge*

The figures refer to mg. N in 100 ml. of the mixture

Period of aeration	Liquid			Moist solid adsorbed
	NH_3-N	NO_2- + NO_3-N	NO_2-N	NH_4-N
At start	4·3	0·0	–	0·3
30 min.	4·2	0·1	+	0·6
2 hr.	3·5	0·6	+ + +	0·4
4 hr.	2·4	1·4	+ + +	0·5
6 hr.	1·4	2·2	+ + +	0·2
8 hr.	0·6	3·4	+ + +	0·2
24 hr.	0·1	3·9	–	0·1

+ Indicates a weak reaction and + + + a strong reaction for nitrite.

agree with those of Heukelekian [1936], show that the amount of ammonium-N adsorbed on the sludge was greatest shortly after addition of fresh sewage and decreased as the treatment proceeded; the amount adsorbed at any time was small.

The evidence for Cambier's conclusion [1920], that adsorption of ammonium ions from sewage by activated sludge may be considerable, rests upon the rapid disappearance of ammonia from sewage, without any corresponding nitrification, after the sewage had been in contact with sludge for 15 min.

Distribution of nitrogenous compounds during aeration of mixtures of sewage and activated sludge

(*a*) *Mixtures in which no nitrification occurred.* In some experiments in which 2-l. mixtures of sewage and activated sludge were aerated in Winchester bottles, there was an approximately exponential diminution in the concentration of the ammoniacal N in the liquid phase, accompanied by a corresponding increase in the N content of the sludge, but no oxidation to nitrite or nitrate occurred (Table 2). The addition of ammonium sulphate before aeration did not alter this type of change during the experiments. When potassium nitrite or nitrate was added to the mixture of sewage and sludge, there was a

Table 2. *Distribution of nitrogenous compounds during aeration of non-nitrifying mixtures of sewage and activated sludge, with added nitrogenous compounds*

The figures refer to mg. N in 100 ml. of mixture

Period of aeration	Liquid					Moist solid Total N	Total N in both phases
	NH_3-N	NO_2-N	NO_3-N	Organic N	Total N		
				(*a*) No addition			
At start	4·3	0·0	0·0	0·2	4·5	31·2	35·7
2 hr.	3·8	0·0	0·0	0·2	4·0	31·1	35·1
5 hr.	3·3	0·0	0·0	0·5	3·8	31·5	35·3
1 day	1·4	0·0	0·0	0·2	1·6	34·0	35·6
2 days	0·2	0·0	0·0	0·3	0·5	35·0	35·5
3 days	0·0	0·0	0·0	0·2	0·2	35·7	35·9
				(*b*) N added in the form of ammonium sulphate			
At start	7·8	0·0	0·0	0·2	8·0	30·9	38·9
2 hr.	7·0	0·0	0·0	0·5	7·5	—	—
5 hr.	6·8	0·0	0·0	0·2	7·0	32·7	39·7
1 day	4·5	0·0	0·0	0·5	5·0	—	—
2 days	3·3	0·0	0·0	0·1	3·4	36·0	39·4
3 days	2·8	0·0	0·0	0·2	3·0	36·3	39·3
6 days	1·8	0·0	0·0	0·3	2·1	36·3	38·4
				(*c*) N added in the form of potassium nitrite			
At start	3·7	2·4	0·0	0·2	6·3	27·6	33·9
2 hr.	3·3	1·0	0·0	0·1	4·4	27·5	31·9
5 hr.	2·9	0·2	0·0	0·1	3·2	28·2	31·4
1 day	0·7	0·0	0·0	0·1	0·8	30·5	31·3
2 days	0·0	0·0	0·0	0·1	0·1	32·0	32·1
3 days	0·0	0·0	0·0	0·0	0·0	32·3	32·3
				(*d*) N added in the form of potassium nitrate			
At start	3·4	0·0	2·5	0·4	6·3	27·9	34·2
2 hr.	3·2	1·1	0·3	0·2	4·8	27·7	32·5
5 hr.	3·1	0·2	0·0	0·2	3·5	28·6	32·1
1 day	0·6	0·0	0·0	0·1	0·7	31·2	31·9
2 days	0·2	0·0	0·0	0·1	0·3	31·8	32·1
3 days	0·0	0·0	0·0	0·1	0·1	31·8	31·9

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rapid diminution, during aeration, in the concentration of oxidized N; only a small portion of this diminution could be accounted for by an increase in the organic N. In these experiments, added nitrite was not converted into nitrate, but nitrite was always detected during aeration of mixtures to which nitrate had been added. The increase in the amount of N in the moist solid was approximately the same as the decrease in the amount of ammonia-N in the liquid

Table 3. *Distribution of nitrogenous compounds during aeration of non-nitrifying mixtures of sewage and activated sludge, with added nitrogenous compounds*

No additions were made in the experiments marked with an asterisk (*). The air entering the system was ammonia-free (passage through sulphuric acid) and the ammonia liberated from the flasks and carried in the air current was collected and estimated: the amount of ammonia-N thus liberated did not exceed 0·2 mg. for any series of flasks except series (*b*).

The figures refer to mg. N per 125 ml. of mixture and, except in series (*b*), are the means of duplicate experiments.

Before aeration					After aeration					
Organic N	NH_3-N	NO_2-N	NO_3-N	Total N	Organic N	NH_3-N	NO_2-N	NO_3-N	Total N	Loss of N
(*a*) N added in the form of ammonium sulphate: 2 days' aeration										
12·4	10·5	0·1	0·0	23·0	14·9	7·9	0·0	0·0	22·8	0·2
12·4	3·7	0·1	0·0	16·2	12·6	3·2	0·0	0·0	15·8	0·4
12·4	2·3	0·1	0·0	14·8	12·4	1·6	0·0	0·0	14·0	0·8
*12·4	1·4	0·1	0·0	13·9	13·1	0·2	0·0	0·0	13·3	0·6
(*b*) N added in the form of ammonium sulphate: 14 days' aeration										
7·6	32·1	0·0	0·0	39·7	9·6	27·0	0·0	0·0	36·6	3·1
7·6	25·9	0·0	0·0	33·5	10·0	23·9	0·0	0·0	33·9	0·4 (gain)
7·6	19·7	0·0	0·0	27·3	10·6	15·4	0·0	0·0	26·0	1·3
7·6	13·4	0·0	0·0	21·0	10·8	9·0	0·0	0·0	19·8	1·2
7·6	7·2	0·0	0·0	14·8	11·3	2·6	0·0	0·0	13·9	0·9
7·6	4·1	0·0	0·0	11·7	11·1	0·1	0·0	0·0	11·2	0·5
*7·6	1·0	0·0	0·0	8·6	8·9	0·0	0·0	0·0	8·9	0·3 (gain)
*7·6	1·0	0·0	0·0	8·6	8·5	0·0	0·0	0·0	8·5	0·1
								Gross loss		6·4
								N liberated at NH_3		6·4
								N unaccounted for		0·0
(*c*) N added in the form of potassium nitrite: 2 days' aeration										
12·3	2·4	8·7	0·0	23·4	11·7	0·9	5·0	0·0	17·6	5·8
12·3	2·4	2·2	0·0	16·9	12·4	0·6	0·5	0·0	13·5	3·4
12·3	2·4	0·9	0·0	15·6	12·9	0·2	0·0	0·0	13·1	2·5
*12·3	2·4	0·0	0·0	14·7	12·2	1·2	0·0	0·0	13·4	1·3
(*d*) N added in the form of potassium nitrite: 14 days' aeration										
14·2		20·4	0·0	34·6	12·4	0·0	0·0	0·9	13·3	21·3
14·2		10·2	0·0	24·4	11·1	0·0	0·0	0·6	11·7	12·7
14·2		4·1	0·0	18·3	10·8	0·0	0·0	0·0	10·8	7·5
*14·2		0·0	0·0	14·2	11·2	0·0	0·0	0·0	11·2	3·0
(*e*) N added in the form of potassium nitrate: 2 days' aeration										
12·1	2·9	0·0	9·0	24·0	12·7	2·1	5·8	0·0	20·6	3·4
12·1	2·9	0·0	2·3	17·3	14·3	2·1	0·0	0·0	16·4	0·9
12·1	2·9	0·0	0·9	15·9	14·1	2·0	0·0	0·0	16·1	0·2 (gain)
*12·1	2·9	0·0	0·0	15·0	12·9	2·2	0·0	0·0	15·1	0·1 (gain)
(*f*) N added in the form of potassium nitrate: 14 days' aeration										
16·0		0·0	66·9	82·9	13·9	0·0	0·0	11·7	25·6	57·3
16·0		0·0	33·5	49·5	13·8	0·1	0·0	5·1	19·0	30·5
16·0		0·0	13·5	29·5	13·4	0·0	0·0	1·3	14·7	14·8
*16·0		0·0	0·0	16·0	15·7	0·0	0·0	0·0	15·7	0·3

(Table 2). In the experiments with sewage alone and with added ammonia-N, the total N in the mixtures remained nearly constant during aeration for 3 days. In the experiments in which nitrite or nitrate was added, the total N decreased rapidly during aeration by an amount approximately equivalent to the decrease in the content of nitrite or nitrate.

In other experiments, mixtures of 25 ml. sewage and activated sludge with 100 ml. water, to which different quantities of nitrogenous compounds had been added, were aerated continuously for several days in 500 ml. flasks, and the distribution of N was determined at the beginning and end of each experiment. Typical results are given in Table 3. In the experiments in which ammonium sulphate was added, the ammonia-N lost from the liquid phase during aeration was converted almost quantitatively into organic N in the sludge, but in Exp. *b* there was a loss of ammonia from the mixtures to which large amounts of ammonium sulphate had been added: some ammonia-N remained in the liquid phase after 14 days. When potassium nitrite or nitrate was added to the mixtures of sewage and activated sludge, there was a loss of N during aeration: the loss appeared to be due to the escape of N_2 and no ammonia was detected in the escaping gases. The amount of N thus lost increased as the concentration of added potassium nitrite or nitrate was increased. In these experiments, no indication of the formation of hyponitrous acid or of any other likely intermediate compound was obtained, except for the production of variable quantities of nitrite with mixtures containing added nitrate. In separate experiments in which mixtures of potassium nitrite, sewage and activated sludge were allowed to stand without aeration but under aerobic conditions the gas evolved was almost pure N_2 and contained no N_2O or other combustible gases.

(*b*) *Mixtures in which nitrification occurred.* The results of experiments in which nitrite or nitrate was formed during aeration of mixtures of sewage and activated sludge, with added nitrogenous compounds, are given in Table 4. The total N content of the mixtures remained approximately constant during aeration in all cases. In mixtures with no added substances or containing added ammonium sulphate, a large proportion of the ammonia-N in the liquid phase was oxidized to nitrate, with the intermediate formation of nitrite. On some occasions, as indicated by the resorcinol-potassium periodate test [Corbet, 1934],[1] there were indications of the formation of small quantities of hyponitrite.

When potassium nitrite was added in an amount approximately equivalent to the ammonia-N already present in the sewage, the concentration of nitrite remained nearly constant during aeration until the whole of the ammonia-N had disappeared from the sewage and had been converted into organic N in the sludge; the nitrite was then oxidized to nitrate. The fact that the synthetic or protein-forming stage normally precedes that of nitrification was known to Adeney in 1895.

Effect of the carbon/nitrogen ratio

Many workers have attempted to explain the results of experiments with sewage or soil in terms of the carbon/nitrogen (C/N) ratio of the system under examination, and it might be suggested that, in the experiments described in this

[1] As Sundara Rao *et al.* [1938] have made certain criticisms against this test, it may be pointed out that it was not claimed that it was "delicate" or "specific", and it was clearly stated that hydroxylamine, borates and bicarbonates behaved similarly. This test is useful for distinguishing between hyponitrite and nitrite as, despite the statement made by the Indian investigators, the latter does not give a positive reaction.

Table 4. *Distribution of nitrogenous compounds during aeration of nitrifying mixtures of sewage and activated sludge, with added nitrogenous compounds*

The figures refer to mg. N in 100 ml. of the mixture

Period of aeration	Liquid					Moist solid Total N	Total N in both phases
	NH_3-N	NO_2-N	NO_3-N	Organic N	Total N		
	(*a*) No addition						
At start	1·5	0·0	0·5	0·5	2·5	13·6	16·1
2 hr.	1·2	0·2	0·4	0·1	1·9	14·6	16·5
5 hr.	0·6	0·4	0·6	0·2	1·8	14·7	16·5
1 day	0·0	0·0	1·3	0·2	1·5	15·1	16·6
2 days	0·0	0·0	1·6	0·3	1·9	14·7	16·6
3 days	0·0	0·0	1·8	0·1	1·9	14·7	16·6
	(*b*) N added in the form of ammonium sulphate						
At start	6·7	0·0	0·0	0·9	7·6	9·6	17·2
2 hr.	6·7	0·1	0·3	0·6	7·7	9·8	17·5
5 hr.	6·7	0·3	0·5	0·3	7·8	9·7	17·5
1 day	5·0	1·2	1·4	0·1	7·7	9·7	17·4
2 days	4·4	1·3	1·9	0·1	7·7	9·6	17·3
3 days	3·4	0·1	4·0	0·2	7·7	9·5	17·2
	(*c*) N added in the form of potassium nitrite						
At start	2·3	2·1	0·0	0·6	5·0	5·7	10·7
2 hr.	2·3	2·1	0·0	0·4	4·8	5·7	10·5
5 hr.	1·5	2·4	0·0	0·4	4·3	6·5	10·8
1 day	0·0	2·2	0·3	0·1	2·6	8·5	11·1
2 days	0·0	0·0	1·9	0·0	1·9	8·4	10·3
3 days	0·0	0·0	2·0	0·0	2·0	8·4	10·4

paper, the extent of nitrification was dependent on the C/N ratio of the mixtures. Some experiments were carried out in which mixtures of sewage and activated sludge were aerated and the effect of alterations in the ratio of organic C to total N in the liquid phase was investigated.

In the first experiments, 2 l. of a mixture of settled sewage and activated sludge were aerated for a period of 23 hr.; the activated sludge was then allowed to settle and the supernatant liquid was siphoned off. Settled sewage, or on some occasions settled sewage mixed with an equal volume of an aqueous solution of glucose, was then added to the sludge remaining in the aeration vessel to bring the volume of the mixture to 2 l., the mixture was stirred and a sample was withdrawn for analysis; the mixture was then aerated for 23 hr. This daily cycle of operations was repeated in one experiment for 27 days and in another for 30 days. The sample withdrawn after each addition of fresh liquid was filtered through filter paper and the quantities of organic C and total N were determined in the filtrate and in the moist solid; in addition, the concentration of ammoniacal N was estimated in the filtrate. Nitrate-N was determined in the supernatant liquid withdrawn after settlement of the activated sludge at the end of each period of aeration. The results obtained are given in Table 5.

In mixtures of settled sewage containing glucose, the C/N ratio was, of course, much higher than in settled sewage alone. The C/N ratio in the activated sludge which had been aerated for several periods of 23 hr. in admixture with diluted settled sewage containing added glucose was, however, not greatly different from the ratio after aeration of the sludge in admixture with settled sewage alone. The C/N ratio of activated sludge thus appeared to be relatively unaffected by the composition of the liquid with which it was aerated. When the sludge

Table 5. *Effect of the carbon/nitrogen ratio of the liquid phase during aeration for 23 hr. of mixtures of activated sludge and sewage or sewage containing added glucose*

Period of aeration (days)	Weight of glucose added to 2 l. of mixture (g.)	Composition of liquid phase from filtration of sample of mixture of activated sludge and sewage before aeration: Organic carbon (mg. per 50 ml.)	Total N (mg. per 50 ml.)	C/N ratio	Composition of moist solid phase (i.e. sludge) from filtration of sample of mixture of activated sludge and sewage before aeration: Organic carbon (mg. per 50 ml.)	Total N (mg. per 50 ml.)	C/N ratio	Composition of mixture of activated sludge and sewage before aeration: Organic carbon (mg. per 50 ml.)	Total N (mg. per 50 ml.)	C/N ratio	Soluble nitrate-N after aeration of mixture for 23 hr. as % of soluble ammonia-N at beginning of aeration
					Experiment A						
1	0	1·2	1·0	1·2	—	10·7	—	—	11·7	—	56
2	0	2·1	1·2	1·8	52·5	10·5	5·0	54·6	11·7	4·7	91
6	1·0	9·4	1·0	9·4	59·9	11·4	5·3	69·3	12·4	5·6	22
8	1·0	13·1	0·9	14·5	71·0	12·2	5·8	84·1	13·1	6·4	0
11	1·0	10·8	0·7	15·4	78·7	13·1	6·0	89·5	13·8	6·5	0
14	1·0	15·3	0·8	19·1	69·9	12·6	5·5	85·2	13·4	6·4	0
17	0	4·4	2·4	1·8	61·2	11·7	5·2	65·6	14·1	4·6	32
20	0	2·0	2·5	0·8	53·4	10·7	5·0	55·4	13·2	4·2	73
23	0	2·6	1·2	2·2	53·6	10·6	5·1	56·2	11·8	4·8	80
27	0	4·7	2·6	1·8	51·9	11·1	4·7	56·6	13·7	4·1	72
							Mean = 5·3				
					Experiment B						
1	0	—	—	—	47·3	7·8	6·1	—	—	—	100
2	0	—	—	—	41·0	6·7	6·1	—	—	—	100
3	0	—	—	—	35·6	5·7	6·3	—	—	—	85
6	0·25	—	—	—	33·8	5·9	5·7	—	—	—	71
8	0·5	—	—	—	32·3	5·8	5·6	—	—	—	100
10	0·5	—	—	—	36·4	7·0	5·2	—	—	—	0
13	1·0	—	—	—	32·3	9·1	3·6	—	—	—	0
15	1·0	—	—	—	61·1	9·7	6·3	—	—	—	0
18	0	—	—	—	68·8	12·2	5·6	—	—	—	64
21	0	—	—	—	59·4	11·5	5·2	—	—	—	67
24	0	—	—	—	51·0	11·0	4·6	—	—	—	77
27	0	—	—	—	41·4	8·4	4·9	—	—	—	80
30	0	—	—	—	47·2	10·0	4·7	—	—	—	85
							Mean = 5·6				

There is no significant difference between the means of the C/N ratio of the moist solids in the two experiments, for $n = 20$, $t = 0{\cdot}876$ and P lies between 0·4 and 0·3 [*v.* Fisher, 1934; Corbet & Wooldridge, 1936]. In the present investigation, differences between means are regarded as significant only when $P < 0{\cdot}01$.

was aerated in admixture with settled sewage alone, a large proportion of the N in the sewage appeared as nitrate after each period of aeration. When settled sewage containing added glucose was used, oxidation to nitrate ceased but began again when settled sewage only was used. This is attributed to the fact that, in the presence of an excess of carbonaceous material available for bacterial growth requirements, soluble ammonia-N is utilized in the formation of insoluble organic nitrogenous compounds in the sludge; on the other hand, any ammonia-N in excess of the available carbonaceous material present is oxidized to nitrate.

Biochemical oxygen demand and bacterial counts

Determinations of the biochemical oxygen demand, by the method described by Wooldridge & Standfast [1933], and of counts of viable bacteria were made on the liquid phase during experiments in which mixtures of sewage and

activated sludge were aerated. An early and rapid O_2 uptake occurred, whether or not nitrate was formed during aeration of the mixtures, and the O_2 uptake was accompanied by a rapid increase in the numbers of bacteria (Tables 6 and 7).

Table 6. *Biochemical oxygen demand, bacterial counts and distribution of nitrogenous compounds in a mixture of sewage and activated sludge during aeration: no nitrate formed*

The figures for N and oxygen refer to mg. per 100 ml. and the bacterial numbers to colonies per 1·0 ml. of liquid.

	Liquid						
Period of aeration	NH_3-N	NO_2-N	NO_3-N	Organic N	B.O.D.	Moist solid Total N	Total N in both phases
Biochemical oxygen demand correlated with the disappearance of ammonia							
At start	2·4	0·0	0·0	0·3	17·2	22·5	25·2
2 hr.	1·6	0·0	0·0	0·4	10·0	23·5	25·5
5 hr.	1·0	0·6	0·0	0·1	3·6	23·8	25·5
1 day	0·4	0·0	0·0	0·1	4·1	25·1	25·6
2 days	0·0	0·0	0·0	0·4	1·2	25·6	26·0

Bacterial counts correlated with the disappearance of ammonia

	Liquid			
Period of aeration	NH_3-N	NO_2-N	NO_3-N	Bacterial counts on heart agar after 48 hr. at 34°
At start	3·8	0·0	0·0	1,120,000
2 hr.	3·7	0·0	0·0	3,575,000
5 hr.	3·4	0·0	0·0	2,046,000
1 day	1·6	0·6	0·0	944,000
2 days	0·2	0·2	0·4	343,000

Table 7. *Biochemical oxygen demand, bacterial counts and distribution of nitrogenous compounds in a mixture of sewage and activated sludge during aeration: nitrate formed*

The figures for the N and oxygen refer to mg. per 100 ml. and the bacterial numbers to colonies per 1·0 ml. of liquid.

Biochemical oxygen demand and bacterial numbers correlated with the disappearance of ammonia

	Liquid				
Period of aeration	NH_3-N	NO_2-N	NO_3-N	B.O.D.	Bacterial counts on heart agar after 48 hr. at 34°
At start	3·1	0·0	0·0	14·0	1,393,000
2 hr.	2·4	0·1	0·4	4·4	7,864,000
5 hr.	1·8	0·1	1·0	2·8	1,027,000
1 day	0·0	0·0	2·3	0·8	309,000
2 days	0·0	0·0	2·8	1·2	58,000

Wooldridge & Standfast [1936] showed that the oxidations effected during treatment of sewage by the activated sludge process were dependent on the presence of bacterial enzymes. In the present investigation, it was found that heat sterilization of the mixtures containing added potassium nitrite arrested the processes of nitrification and protein synthesis (Table 8).

Table 8. *Distribution of nitrogenous compounds during aeration of sterilized mixtures of sewage and activated sludge, containing added potassium nitrite*

The figures refer to mg. N contained in the flasks
N added in the form of potassium nitrite: 5 days' aeration

Before aeration				After aeration				
Organic and NH_3-N	NO_2-N	NO_3-N	Total N	Organic and NH_3-N	NO_2-N	NO_3-N	Total N	Loss of N
4·7	0·1	0·1	4·9	4·0	0·0	0·1	4·1	0·8
4·7	0·1	0·1	4·9	3·5	0·1	0·1	3·7	1·2
4·7	2·3	0·1	7·1	4·1	2·4	0·0	6·5	0·6
4·7	4·4	0·1	9·2	4·0	4·5	0·1	8·6	0·6
4·7	6·6	0·1	11·4	4·8	6·1	0·0	10·9	0·5
4·7	8·7	0·1	13·5	4·3	8·3	0·0	12·6	0·9

Effect of the concentration of ammonia-nitrogen on the changes during aeration of mixtures of sewage and activated sludge

As the two main reactions discussed in this paper have ammonia as an initial reactant, it was important to determine whether the concentration of ammonia present at any time favoured one reaction while inhibiting the other. Meyerhof [1916, 1917] showed that the oxidation of ammonia to nitrite and of nitrite to nitrate, in the presence of nitrite- or nitrate-forming bacteria, only takes place below certain limiting concentrations of ammonium ($0{\cdot}1N$), or nitrite ($>0{\cdot}5N$) ions respectively. In the experiments described in this paper, the concentration of ammonia-N never exceeded the value which, according to Meyerhof, would permit formation of nitrite to proceed at 70 % of the optimum rate. Moreover, in two parallel experiments in which equal amounts of ammonium sulphate were added to mixtures of sewage and activated sludge, in one experiment at the beginning of the period of aeration and in the other experiment in small instalments during aeration over a period of several days, the distribution of the N compounds at the end of the experiments was approximately the same. It is clear, therefore, that in the experiments in which no nitrification occurred during aeration of mixtures of sewage and sludge, the concentration of ammonium ions was not responsible for the absence of nitrification. Nevertheless it appears that concentrations of ammonia-N even below the limiting value found by Meyerhof can exert a marked inhibitory effect on the course of the reactions concerned with synthesis of protein. Table 9, which has been prepared

Table 9. *Relation between the concentration of ammonium-nitrogen in sewage and its conversion into organic nitrogen*

The figures are calculated from the data given in Table 3 (*b*)

	NH_3-N in liquid in mg. per 125 ml. of mixture						
	1·0	4·1	7·2	13·4	19·7	25·9	32·1
% of NH_3-N in liquid phase, converted to organic form	100·0	85·4	51·4	23·9	15·2	9·3	6·2
Increase in organic N in mixture in mg.	1·0	3·5	3·7	3·2	3·0	2·4	2·0

from the data given in Table 3 (*b*), shows that after aeration of a mixture of sewage and sludge for 14 days, the whole of the ammoniacal N was transformed into organic form only in the mixtures containing no added ammonium salt.

In the mixture in which the greatest increase occurred in the amount of organic N, only about 50 % of the ammoniacal N in the liquid phase was converted into organic form. In this experiment the excess of ammoniacal N not converted into organic form remained unaltered in the liquid and was not oxidized to nitrite or nitrate.

Knight [1936] has pointed out that amino-acids strongly inhibit the respiration and proliferation of *Nitrosomonas* and *Nitrobacter*. Amino-acids were not detected in measurable amounts during the present investigation when mixtures of sewage and activated sludge were aerated for periods up to 13 days. When Beesley's medium[1] was inoculated with small amounts of activated sludge, nitrite was always produced, showing that organisms capable of converting ammonia into nitrite were present, even in mixtures of sewage and activated sludge in which no nitrification occurred during aeration. Nitrite was also formed when Beesley's medium containing appreciable amounts of sterilized sludge was similarly inoculated. The inhibition of nitrification during aeration of some of the mixtures was thus not due to the absence of the appropriate organisms, or to the presence of excessive quantities of organic matter in the sludge; nor could it have been due to O_2 lack.

Methods of analysis

Ammonia-N was estimated by distillation into standard acid. The sum of the nitrite- and nitrate-N was obtained by the method entailing simultaneous reduction with Devarda's alloy and distillation of the ammonia thus formed into standard acid. Nitrite-N was determined by the Griess-Ilosvay colorimetric method. In the absence of nitrite or nitrate, estimation of the ammonia- and organic-N was effected on a separate sample by the usual modified Kjeldahl method, involving the addition of potassium and copper sulphates.

Tests showed that when nitrite was present, the total N in sewage material could not be estimated by pre-treatment with reduced iron and sulphuric acid, followed by the usual digestion by the modified Kjeldahl method. Analysis of mixtures of sewage and sludge containing nitrite and/or nitrate was effected satisfactorily in the following manner. The mixture was filtered and the organic N in the well-washed solid was determined by the modified Kjeldahl method; on an aliquot portion of the filtrate were estimated first the ammonia, then the sum of the nitrite- and nitrate-N (by reduction with Devarda's alloy) and, finally, the total N. When only small amounts of soluble organic nitrogenous material were present in the liquid phase, as was usually the case, the magnesia and Devarda's alloy were removed by filtration, after distillation, and the filtrate was analysed for organic N as usual: when organic N was present in quantity, the Kjeldahl digestion was carried out with the whole of the mixture remaining after distillation with Devarda's alloy.

Summary

An experimental study has been made of the distribution of N compounds during treatment of sewage by the activated sludge process.

In some experiments, part of the ammoniacal N in the liquid phase was transformed into organic form in the sludge during aeration of the mixture; the remainder of the ammoniacal N was oxidized, usually at a later stage, to

[1] Beesley's medium contains 0·95 g. $(NH_4)_2SO_4$, 0·2 g. KH_2PO_4, 0·2 g. NaCl, 0·066 g. $MgSO_4$, $7H_2O$, 20·0 g. $CaCO_3$, and 1 l. distilled water.

nitrite and/or nitrate and remained in solution in the liquid. There was little or no loss of gaseous N.

In other experiments the whole of the ammoniacal N in the liquid was converted into organic form in the sludge during aeration. When nitrite or nitrate was added to such non-nitrifying mixtures of sewage and sludge, large losses of gaseous N_2 occurred.

Nitrification was inhibited when glucose was added to the sewage, but began again shortly after addition of glucose was discontinued. The carbon/nitrogen ratio of the activated sludge was relatively unaffected by such addition of glucose with the sewage.

The absence of nitrification was not due to the absence of the appropriate micro-organisms, or to the presence of excessive amounts of ammonia-N.

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124. THE NITROGEN CYCLE IN BIOLOGICAL SYSTEMS

2. CHANGES IN MIXTURES OF SEWAGE AND ACTIVATED SLUDGE ENTAILING THE LIBERATION OF NITROGEN

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Some of the early work on the biological aspects of sewage purification was concerned with the nitrification processes. Mair [1908] considered the problem with special reference to denitrification, using a percolating filter in combination with contact beds. He detected hyponitrous acid during the reduction of nitrate in sewage and isolated an organism, *Bacillus hyponitrosus*, able to reduce nitrate to hyponitrite in pure culture. Mair observed also that the disappearance of nitrate, which occurred at one stage of the treatment, was accompanied by the release of N_2 and he regarded this as an instance of denitrification. Some investigators, e.g. Heukelekian & Chamberlin [1922–3] and Bach [1932], have reported losses of N during the treatment of sewage by the activated sludge process. Barritt [1931; 1933] discussed nitrification in biological filters and reviewed the literature on the subject of the liberation of N by bacteria. Bonazzi [1921] found that *Azotobacter chroococcum* effected fixation of N in a medium with or without the addition of ammonium sulphate, but a loss of N resulted from the utilization of nitrate by this organism. Korsakova [1927] showed that, during the oxidation of such carbon compounds as glucose and citric acid by *B. fluorescens liquefaciens* or by *B. pyocyaneus*, the formation of protein from the N of nitrate was accompanied by a liberation of N_2 and the intermediate production of nitrite. Richards & Shrikhande [1935], who investigated the utilization of different forms of inorganic N during the decomposition of plant materials, showed the possibility of denitrification during the conversion of nitrate- into protein-N.

In the previous paper [Corbet & Wooldridge, 1940], it was shown that, under certain conditions, and particularly with non-nitrifying mixtures of sewage and activated sludge, N was lost as gas during aeration. In this paper the problem of liberation of N is examined further, using fresh activated sludge, sterilized sludges subsequently inoculated with organisms in pure culture and chemically defined media inoculated with organisms.

Experimental technique

The technique of analysis was similar to that described in the previous paper. When necessary, the sewages and sludges and the chemically defined media were sterilized by autoclaving. A medium frequently used was Reader's

medium,[1] sometimes modified in the ways described in the text. Various micro-organisms were used but *Chromobacterium prodigiosum* and a yeast isolated from sewage were often employed. The characteristics of this yeast were studied and, as no ascospore formation was observed, it has been assigned to the genus *Torula*. This generic classification is tentative and, for purposes of reference, it is proposed to name the organism Torula nitrivertens *sp.nov.*

This organism,[2] which is a bottom yeast, grew well on the usual media, good growth being obtained on beer-wort agar overnight. The organism was obtained in pure culture by continued subculturing in beer-wort broth adjusted to *p*H 2·8. A thorough and repeated examination by microscopic methods (stained and unstained preparations), and by cultivation on ordinary bacteriological media, failed to reveal the presence of any bacterial contaminants. Unstained, this organism had the appearance of a typical yeast, comprising large, ellipsoid cells, which varied somewhat in size and shape according to the conditions of cultivation; chains with up to four or five cells were not rare. A stroke culture on beer-wort agar gave abundant growth. With stroke cultures on ordinary nutrient agar, and on nitrate-glucose agar, growth was moderate to abundant, beaded, convex, glistening, smooth, opaque, buff, slimy; no aerial growth. The agar colonies were round, with surface smooth and convex, and edges entire; no liquefaction of gelatin. Growth in heart broth and in nitrate-glucose solution good; in Thornton's agar (an inorganic salt mixture containing asparagine) very poor, only becoming evident after 4 or 5 days. Facultative anaerobe. No chromogenesis. After incubation for 3 days at 34°, the organism produced acid and gas with sucrose; acid and a little gas with glucose; no change with maltose, lactose and mannitol; and only slight curd formation with litmus milk. Nitrate was reduced to nitrite. It will be seen that this organism was able to liberate or fix N under the appropriate conditions. It is not, however, the first N-fixing yeast to be reported, for de Kruijff [1910] described the isolation from garden soil in Java of such an organism, which he named *Torula bogoriensis rubra*, since it formed blood-red colonies.

Experiments with sterilized mixtures of sewage and activated sludge inoculated with pure cultures of micro-organisms

When sterilized mixtures of sewage and activated sludge were inoculated with pure cultures of different organisms and were incubated for about 2 weeks at 34° there was often a significant loss of N, although no loss occurred from the uninoculated controls (Table 1). Thus, it is evident that a mixed flora is not essential to a loss of N from sewage systems. The addition of other carbon- or N-containing substances may affect the ultimate distribution of the N. The experiments given in Table 2 indicate that the loss of N from non-sterile mixtures of sewage and activated sludge can be largely or completely annulled by the addition of such substances as glucose and pyruvic acid, whereas the loss may be increased by the addition of nitrate or glycine. Sterilized mixtures of sewage and sludge inoculated with *Chromobacterium prodigiosum*, *Torula nitrivertens* or *Staphylococcus aureus* showed no significant loss of N in presence of sucrose, in contrast to the significant losses which occurred with potassium nitrate (Table 3).

[1] Reader's medium contains 3 g. $(NH_4)_2SO_4$; 1 g. KH_2PO_4; 0·16 g. K_2HPO_4; 0·5 g. NaCl; 0·7 g. $MgSO_4$, $7H_2O$; 0·4 g. $CaCl_2$, $6H_2O$; 50 g. glucose and 1000 ml. distilled water.

[2] We are indebted to Miss R. Fellowes for help with the isolation of this organism.

Table 1. *Losses of N during incubation of sterilized mixtures of sewage and activated sludge inoculated with cultures of different species of micro-organisms*

The mixtures were incubated at 34° for 14 days (top table) and for 17 days (lower table).

The figures refer to mg. N in 25·0 ml. of sterilized sewage and sludge; each figure represents the mean for four flasks.

Micro-organism	Initial N content	Final N content	% loss of N	Significance of difference between initial and final N contents* n	t	P
Uninoculated controls	4·5	4·5	—	6	—	—
Staphylococcus albus	8·5	8·0	5·9	6	4·62	<0·01
Chromobacterium prodigiosum	8·4	7·0	16·7	6	10·33	<0·01
Pseudomonas pyocyanea	8·3	7·2	13·3	6	9·53	<0·01
Torula nitrivertens	8·6	8·3	3·5	6	4·24	<0·01
Uninoculated controls	4·6	4·7	—	6	0·49	Between 0·7 and 0·6
Undetermined short rod, isolated from sewage	5·8	4·7	19·0	6	5·88	<0·01

* See Corbet & Wooldridge [1936]. $n=(N-1)+(N'-1)$, N and N' being the number of replicate determinations.

$$t=\frac{\bar{x}-\bar{x}'}{s}\sqrt{\frac{1}{N}+\frac{1}{N'}},$$

$\bar{x}$ and $\bar{x}'$ being the respective means and s the estimated standard deviation.

P represents the probability of the difference between the means occurring by chance. In the present investigation, differences between means are regarded as significant only when $P<0{\cdot}01$, that is, when such differences can occur by chance less than once in a hundred times.

Table 2. *Changes in the N content of mixtures of sewage and activated sludge containing added compounds, during incubation for 45 days at 29°*

The figures in the second and third columns are means of four experiments and refer to mg. N per 125 ml. of sewage and sludge mixture.

Compounds added	Initial N content	Final N content	% loss	Difference between initial and final N values n	t	P
No addition	6·3	4·6	27·0	6	13·52	<0·01
0·25 g. glucose	6·3	5·8	7·9	6	2·87	Between 0·05 and 0·02
0·50 g. glucose	6·3	6·5	3·2 (gain)	5	1·00	Between 0·4 and 0·3
0·05 g. pyruvic acid	6·3	5·2	17·5	6	5·50	<0·01
0·10 g. pyruvic acid	6·3	6·6	4·8 (gain)	6	2·27	Between 0·1 and 0·05
0·0060 g. glycine	12·3	6·6	46·3	5	20·70	<0·01
0·0120 g. glycine	18·3	7·4	59·6	6	43·02	<0·01
0·0043 g. NO_3-N in KNO_3	10·6	4·1	61·3	6	29·54	<0·01

The addition of a small inoculum of a mixture of sewage and activated sludge into Reader's medium resulted in a small loss of N, but the loss was materially increased when the ammonium sulphate (one constituent of the medium) was replaced by potassium nitrate (Table 4). Similarly, although incubation of Reader's medium inoculated with *Chromobacterium prodigiosum* or with *Torula nitrivertens* did not result in a loss of N, there was a loss of N during incubation of Reader's medium inoculated with these organisms when

Table 3. *Changes in the N content of sterilized mixtures of sewage and activated sludge containing added compounds, inoculated with pure cultures of micro-organisms*

The flasks were incubated at 34° for 17 days with *Staph. aureus*, and for 20 days with the other organisms; each test was carried out in quadruplicate. The N content is in mg. per 25·0 ml. of sterilized sewage and sludge mixture.

Addition	Initial N content	Final N content	Significance of difference between initial and final N contents		
			n	t	P
Staphylococcus aureus					
Uninoculated controls	8·5	8·2	6	1·17	Between 0·3 and 0·2
No addition	9·0	9·2	6	0·71	0·5
0·50 g. sucrose	9·0	9·1	6	0·32	Between 0·8 and 0·7
Potassium nitrate (8·3 mg. NO_3-N)	17·3	13·5	6	22·56	<0·01
Chromobacterium prodigiosum					
No addition	14·4	11·6	6	28·00	<0·01
0·48 g. sucrose	14·4	13·9	6	2·47	Between 0·05 and 0·02
Potassium nitrate (8·8 mg. NO_3-N)	23·2	14·2	6	39·26	<0·01
Torula nitrivertens					
0·48 g. sucrose	14·3	14·6	6	0·95	Between 0·4 and 0·3
Potassium nitrate (6·7 mg. NO_3-N)	21·0	19·9	6	6·73	<0·01

Table 4. *Changes in the N content of Reader's medium, inoculated with sewage and activated sludge*

The flasks were incubated for 10 days at 34°.

The figures in the first and second columns refer to mg. N in 25·0 ml. of Reader's medium inoculated with 1·0 ml. of sewage and activated sludge, and represent the mean value of replicated experiments.

Form of N	Initial N content	Final N content	% loss	Significance of difference between initial and final mean values of N		
				n	t	P
Ammonium sulphate	16·8	16·4	2·4	10	5·52	<0·01
Potassium nitrate	12·2	10·3	15·6	10	4·71	<0·01

the ammonium sulphate had been replaced by potassium nitrate (Tables 5 and 6). It will be seen in Table 5 that a small gain in N resulted in the medium containing ammonium salt. Several unsuccessful attempts were made to obtain N fixation in larger quantities, but in an experiment in which 25·0 ml. of Reader's medium containing 0·5 g. of different carbohydrates were inoculated with *Chromobacterium prodigiosum*, after 16 days at 34°, the N content increased from 11·4 to 11·9 mg. (glucose), 11·6 (fructose) and 11·9 (sucrose): in a parallel experiment with *Torula nitrivertens*, the N content increased from 11·4 to 11·7, 11·9 and 11·8 mg. respectively.

Experiments with a pure culture activated sludge

Butterfield [1937] prepared pure culture activated sludges comprising a single bacterial species; these sludges achieved a high rate of oxidation, but no account was taken of the N cycle. We carried out a few experiments with a pure culture sludge, prepared by aeration of a mixture of sterilized sewage and a little sterilized sludge, after inoculation with *Chromobacterium prodigiosum*;

Table 5. *N changes during incubation of Reader's medium, inoculated with* Chromobacterium prodigiosum

The figures refer to mg. N in 25·0 ml. of medium, and each result represents the mean N content of four flasks.

(*a*) Reader's medium with ammonium sulphate[1]

(i) Initial N content	(ii) N content after 14 days at 34°	(iii) N content after 21 days at 34°
34·1	34·4	34·8

Between (i) and (ii), $n=6$, $t=2{\cdot}22$, P between 0·1 and 0·05.
Between (i) and (iii), $n=6$, $t=3{\cdot}74$, $P<0{\cdot}01$.

(*b*) Reader's medium with potassium nitrate

(i) Initial N content	(ii) N content after 21 days at 34°
14·3	9·8

Between (i) and (ii), $n=6$, $6=5{\cdot}88$, $P<0{\cdot}01$.

The flasks contained 7·5 mg. nitrate-N at the beginning of the experiment, and 6·6 mg. nitrite- and nitrate-N at the close, nitrite being present in all four flasks.

Table 6. *Changes in the distribution of N compounds in Reader's medium inoculated with* Torula nitrivertens *during incubation at 34°*

The figures refer to mg. N in 25·0 ml. of medium

(*a*) Reader's medium with ammonium sulphate

Days	Organic N	NH_3-N	NO_2-N	NO_3-N	Total N	Gain (+) or loss (−) of N
—	0·0	11·3	0·0	0·0	11·3	—
1	1·0	10·3	0·0	0·0	11·3	—
4	1·7	9·6	0·0	0·0	11·3	—
8	2·0	8·8	0·0	0·0	10·8	−0·5
11	3·8	8·0	0·0	0·0	11·8	+0·5
15	3·7	8·1	0·0	0·0	11·8	+0·5
23	3·8	8·0	0·0	0·0	11·8	+0·5
28*	3·7	8·2	0·0	0·0	11·9	+0·6
45	4·2	7·5	0·0	0·0	11·7	+0·4

* On the 25th day, 10·0 ml. of sterilized 5% glucose solution were added to each of the remaining two flasks.

(*b*) Reader's medium with potassium nitrate

Days	Organic N	NH_3-N	NO_2-N	NO_3-N	Total N	Loss of N
—	0·2	0·0	0·0	7·9	8·1	—
2	2·1	0·0	0·1	5·6	7·8	0·3
3	1·4	0·0	0·0	4·9	6·3	1·8
7	2·0	0·0	0·0	4·1	6·1	2·0
9*	2·1	0·0	0·0	5·0	7·1	1·0
16	3·2	0·0	0·0	3·2	6·4	1·7
22	3·7	0·0	0·0	3·1	6·8	1·3

* On the 7th day, 10·0 ml. of a sterilized 5% glucose solution were added to each of the remaining three flasks.

¶In a parallel experiment, the gas evolved after 3 days' incubation in an apparatus in which one limb was open to the air was collected by displacement and analysis showed it to consist of 24% N and 76% CO_2.

aeration was continued for several weeks through a number of changes of sterilized sewage. In a subsequent experiment, the purification of sterilized sewage by the small volume of *Chr. prodigiosum* sludge thus obtained was

[1] To ensure good growth, this medium was prepared to contain 1% of peptone.

followed by determination of the changes in the N distribution and in the biochemical oxygen demand (Table 7). This experiment, which was carried out in a 1 l. flask, was conducted as in Table 2 of the previous paper, except that 50·0 ml. samples were taken for the N analysis and B.O.D. determinations at the intervals stated. As the organism used was not a nitrifier, no nitrification occurred of any ammoniacal N present in excess of synthetic requirements.

Table 7. *Treatment of sewage by aeration in admixture with a* Chromobacterium prodigiosum *sludge*

The figures are calculated as mg. N or (in the case of B.O.D.) oxygen in 100 ml. of the mixture (that is, the figures refer to parts per 100,000).

	Supernatant liquid						
Period of aeration	NH_3-N	NO_2-N	NO_3-N	Organic N	B.O.D.	Moist solid Total N	Total N in both phases
Beginning	3·1	0·0	0·0	0·5	18·4	1·0	4·6
4 hr.	2·7	0·0	0·0	0·9	18·2	0·7	4·3
1 day	2·3	0·0	0·0	1·1	13·6	1·1	4·5
2 days	1·7	0·0	0·0	0·9	9·6	0·9	3·5
5 days	1·8	0·0	0·0	1·1	12·7	1·6	4·5

During the 5 days' aeration 2·0 mg. of ammonia-N were liberated. A calculation showed that the contents of the aeration flask (430 ml.) contained 19·8 mg. of N initially, of which 10·7 mg. were removed in samples and 2·0 mg. as ammonia-N, and that no losses of N_2 had occurred.

In a further experiment, mixtures of *Chr. prodigiosum* sludge and sterilized sewage were aerated in a series of glass flasks with additions of ammonium sulphate, potassium nitrite or potassium nitrate (Table 8). In the control

Table 8. *Effect on sewage of aeration in admixture with a* Chromobacterium prodigiosum *sludge, in the presence of added nitrogenous salts*

The incoming air was ammonia-free (passage through sulphuric acid), and the ammonia-N liberated from the sewage material in the flasks during the 5 days' aeration was collected and estimated.

The figures refer to mg. N contained in the flasks and each figure represents the mean of two experiments.

Form of N added to 100·0 ml. sterilized sewage and 10·0 ml. of *Chr. prodigiosum* sludge	Before aeration					After aeration					
	NH_3-N	NO_2-N	NO_3-N	Organic N	Total N	NH_3-N	NO_2-N	NO_3-N	Organic N	Total N	Loss of N
No addition	2·5	0·4	0·0	3·3	6·2	0·4	0·1	0·0	4·7	5·2	1·0
Ammonium sulphate (5·2 mg. N)	7·7	0·4	0·0	3·3	11·4	2·8	0·0	0·0	6·2	9·0	2·4
Potassium nitrite (5·0 mg. N)	2·5	5·4	0·0	3·3	11·2	0·2	4·5	0·0	4·9	9·6	1·6
Potassium nitrate (4·0 mg. N)	2·5	0·4	4·0	3·3	10·2	0·7	0·2	3·6	4·3	8·8	1·4
									Total loss of N		6·4
									N liberated as NH_3-N		1·5
									N presumed lost as gas		4·9

flasks without any additions, there was a conversion of ammoniacal N into organic form together with some loss of N. The addition of ammonia-N resulted in a greater production of protein and a loss of some ammonia in the air stream: the added nitrite or nitrate failed to increase the amount of organic N produced. The

results obtained in these experiments with a pure culture sludge are in accord with the work already described with natural sludges, under conditions in which no nitrification took place.

Rising of activated sludge

Normally, in an activated sludge plant the particles of sludge can readily be settled in a sedimentation tank, but, on occasion, they may rise to the surface, being buoyed up by bubbles of gas which have been found to be N [O'Shaughnessy & Hewitt, 1935]. A number of investigators [e.g. McLachlan, 1936, and Bragstad & Bradney, 1935] observed that rising of sludge was accompanied by the presence of nitrite.

Our experience, which is based upon data from laboratory experiments, has been that nitrite formation can occur with both nitrifying and non-nitrifying mixtures of sewage and sludge. With the former, oxidation from nitrite to nitrate is usually rapid, but, if it is sufficiently slow to allow an accumulation of nitrite, rising of sludge may take place on the cessation of aeration. In these circumstances rising of sludge is not the result of an anaerobic denitrification.

Occasionally, a non-nitrifying sewage and sludge mixture may give rise to nitrite, followed by an accumulation of this compound and rising of the sludge. Tests have been carried out showing that, with such a non-nitrifying mixture, rising can be initiated or aggravated by addition of nitrite or nitrate, or inhibited by addition of assimilable carbonaceous material (glucose or soluble starch). Our results suggest that the occurrence of rising is dependent upon nitrite formation being in progress while some organic matter remains in the sewage, for it appears that N liberation occurs principally during the utilization of nitrite for protein synthesis.

Chemical reactions entailing the decomposition of nitrite

Although small losses of N may occur in sewage and sludge mixtures during aeration, or on prolonged incubation, such losses are usually slight. As the considerable N losses reported in these papers were almost invariably associated with the presence of nitrite and, in some instances, were proved to be due to the release of elementary N, experiments were carried out to ascertain to what extent any loss of N might be due to chemical decomposition of nitrite.

Continuous aeration of mixtures of sewage and sludge containing added nitrite, under conditions of sterility, produced very little disruption of the nitrite, although there was a slight loss of N from the sewage and sludge (Table 8 of previous paper). Similarly, the presence of added ammonia- and nitrite-N together did not result in a loss of nitrite if the conditions were maintained sterile (Table 9).

Except under acid conditions, where there is a chemical oxidation of nitrite to nitrate, the extent of which depends upon the *p*H value of the medium [Corbet, 1934], ammonium nitrite is stable and can be preserved in the dark for some months without change [Corbet, 1935]. In the present investigation, it was found that no loss of N gas occurred when ammonium sulphate and potassium nitrite were boiled together with magnesia and Devarda's alloy, the nitrite being reduced quantitatively to ammonia. The loss of N as ammonia from the sewages during aeration by purely chemical agency was usually negligible. The *p*H value of the mixtures of sewage and sludge was generally between 7·5 and 8·0. While it may be conceded that any available method of sterilizing sewage and sludge may alter their physical and chemical properties to some extent,

Table 9. *Losses of N during the aeration of non-nitrifying mixtures of sewage and sludge, containing added ammonium sulphate and potassium nitrite, under sterile and non-sterile conditions*

The experiments were carried out in a series of flasks as detailed in Table 3 of the previous paper. In the experiment (*a*) the contents of the flasks were found to be sterile at the end of the experiment.

The figures refer to mg. N contained in the flasks.

Before aeration					After aeration					
Organic-+NH_3-N	Added NH_3-N	NO_2-N	NO_3-N	Total N	Organic N	NH_3-N	NO_2-N	NO_3-N	Total N	Loss of N
(*a*) Sterilized sewage and sludge mixtures with N added in the form of ammonium sulphate (5·7 mg.) and potassium nitrite (7·8 mg.): 2 days' aeration										
12·6	5·7	7·8	0·0	26·1	11·0	6·4	7·8	0·0	25·2	0·9
12·6	5·7	7·8	0·0	26·1	11·0	6·8	7·8	0·0	25·6	0·5
12·6	5·7	7·8	0·0	26·1	11·0	6·7	7·8	0·0	25·5	0·6
12·6	5·7	7·8	0·0	26·1	11·0	6·5	7·8	0·0	25·3	0·8
								Total loss		2·8
								N liberated as NH_3		1·7
								N presumed lost as gas		1·1
(*b*) Biologically active sewage and sludge mixtures with N added in the form of ammonium sulphate and potassium nitrite: 5 days' aeration										
6·4	0·0	4·2	0·0	10·6	2·8	2·5	3·1	0·0	8·4	2·2
6·4	0·0	4·2	0·0	10·6	2·8	0·7	3·0	0·0	6·5	4·1
6·4	3·2	4·2	0·0	13·8	2·4	3·7	3·4	0·0	9·5	4·3
6·4	6·3	4·2	0·0	16·9	2·6	6·2	3·2	0·0	12·0	4·9
6·4	9·5	4·2	0·0	20·1	2·8	7·6	2·5	0·0	12·9	7·2
6·4	12·6	4·2	0·0	23·2	2·8	7·6	0·8	0·0	11·2	12·0
6·4	18·9	4·2	0·0	29·5	2·3	17·4	3·1	0·0	22·8	6·7
6·4	25·2	4·2	0·0	35·8	3·0	20·0	0·4	0·0	23·4	12·4
								Total loss		53·8
								N liberated as NH_3		29·0
								N presumed lost as gas		24·8

there is nothing to suggest that heat sterilization would render the sewage substance less reactive chemically. There appears to be no evidence to support the belief that the losses of N reported in these papers were due to chemical reactions involving nitrite. While investigating the chemical aspects of denitrification, Fowler & Kotwal [1924] reached the conclusion that losses of N_2 in nature, in agricultural operations, or in sewage purification, were negligible as far as the chemical reactions they studied were concerned. They were unable to effect any chemical decomposition of ammonium nitrite, by maintaining solutions for 5 hr. at 50° (0·15% NH_4NO_2 and below), and at 96° (0·014% NH_4NO_2 and below), by the action of CO_2 or by certain other specified means.

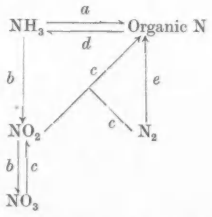
Although we obtained slight losses of N even with sterilized sewage and sludge mixtures, in the presence of added nitrite, these losses were always very much smaller than those found with non-sterile mixtures. Larger losses were obtained during aeration of mixtures of sterilized sewage and sludge to which sterile glycine and nitrite had been added, and this raised the question as to whether the N losses in the non-sterile mixtures were due to reactions involving amino-acids. Frequent determinations during aeration experiments with sewage and sludge mixtures, however, failed to detect amino-acids in any significant amount and it is concluded that the losses of N in our experiments were not to any measurable extent associated with amino-acids but were primarily due to the action of micro-organisms, or their enzymes, upon nitrite under the conditions prescribed.

Discussion

It was formerly believed that denitrification in such biological systems as sewage and soil was largely, if not entirely, an anaerobic process, although data obtained by Richards & Sawyer [1922], Reddie [1925], O'Shaughnessy & Hewitt [1935] and other investigators indicate that considerable quantities of N may be liberated during the aerobic decomposition of sewage under certain conditions.

The experiments described in these papers show that N may be lost from mixtures of sewage and sludge under a variety of conditions, and the evidence suggests that these losses occur as the result of biological activity and that they take place through the intermediate formation of nitrite. No significant losses of N were obtained in our experiments with nitrifying mixtures of sewage and sludge. The results suggest that there are many species of micro-organisms which not only possess the enzymes necessary to reduce nitrate to nitrite but are able to effect the further reduction of nitrite to N_2. The course of the changes involved in the N cycle in experiments with pure organisms varies with the chemical nature of the environment in a manner similar to that with unsterilized mixtures of sewage and activated sludge.

The experiments described in these papers indicate that the path followed by the N depends upon two main factors; the chemical nature of the liquid phase or sewage and the nature of the enzymic mechanisms in the sludge. Activated sludges appear to contain the enzymes necessary to effect any of the changes in the following diagram:



The particular course of reactions followed varies according to the conditions which obtain, and especially as regards the proportions of available carbonaceous matter and N present and the nature of the compound in which the N occurs. The above diagram is intended to be indicative of the possible general reactions and not to show the precise route of the reactions. The reactions indicated in this diagram are encouraged by the following chemical conditions:

(*a*) The presence of ammonia and assimilable carbon compounds.

(*b*) Ammonia present but little or no assimilable carbon compounds.

(*c*) Assimilable carbon compounds present together with nitrite and/or nitrate as the most readily available source of N.

(*d*) Autolytic changes arising from absence of nutrient materials.

(*e*) Fixation of N in the presence of assimilable N-free organic compounds and no readily available source of N other than free gas.

The intermediate mechanism of N fixation is still obscure, but hydroxylamine and oxime formation may be involved as suggested by Virtanen & Laine [1938].

SUMMARY

Sterilized mixtures of sewage and activated sludge inoculated with various species of micro-organisms may lose N, presumably as gas, during incubation.

This loss of N can be reduced or annulled by the addition of assimilable N-free organic compounds, such as various carbohydrates or pyruvic acid; or it may be increased by the addition of nitrate.

In addition to experiments with sterilized sewage and sludge, *Chromobacterium prodigiosum* and *Torula nitrivertens* sp.nov., a yeast isolated from sewage, and other micro-organisms have been used in experiments with a medium of known chemical composition, viz. Reader's medium. If the ammonia of this medium be replaced by nitrate a loss of N results from the growth of these organisms.

A pure culture activated sludge, prepared from *Chromobacterium prodigiosum*, reduced the B.O.D. and ammonia-N content of sterilized sewage.

It has been shown that rising of sludge, which is accompanied by nitrite formation, is encouraged by the addition of nitrite or nitrate and arrested by the presence of readily assimilable N-free organic material, such as glucose or starch.

It is suggested that while activated sludges normally contain all the requisite enzymes for the N changes discussed, the actual course followed depends upon the chemical nature of the liquid phase and the manner in which this influences the rates of the various possible enzymic reactions.

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125. THE NITROGEN CYCLE IN BIOLOGICAL SYSTEMS

3. AEROBIC DENITRIFICATION IN SOILS

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(*Received 29 April 1940*)

ALTHOUGH it was formerly considered that denitrification in soil was largely an anaerobic process, considerable losses of N from tropical soils, under aerobic conditions, have been reported by, e.g., Meggitt [1923], Annett *et al.* [1928], Wilshaw [1934] and Diamond [1937]. Shutt [1910] showed that large losses of N followed cultivation of Canadian prairie soils, and Buswell & Neave [1927] give a detailed bibliography of the literature on the subject of N losses from soil as a result of denitrification. It can scarcely be doubted that, under suitable conditions, aerobic losses of N_2 from soils in temperate regions occur.

Various explanations have been advanced to account for the large N losses which may take place in tropical soils, particularly when primeval forest is felled and the soil is left exposed to the sun. In these latter circumstances, it is usually supposed that the increased temperature consequent upon the removal of shade results in an acceleration of the normal biological processes involving the decomposition of soil organic matter. There is little evidence to support this explanation, however, and in Wilshaw's pot experiments, the N recovered as nitrate or ammonium salts by leaching with water was less than 13 % of the total lost, and the author considered it probable that enormous losses of N_2 occurred. There appears to be no experimental support for the view that soluble nitrogenous material may be leached from the soil in other than insignificant amount.

Losses due to physical changes. We obtained no appreciable losses of N from soil samples submitted to the following experimental conditions:

(1) Exposure of soil to a relatively high temperature in an atmosphere saturated with water vapour did not result in the formation of measurable amounts of soluble N compounds (inorganic or organic) which could be removed by leaching. Over a period of 201 days, the percentage of N changed from 0·174 to 0·165 at 4° and from 0·174 to 0·166 at 32°. (The standard error in all cases was 0·001.) Washing with water failed to effect any significant alteration in the N content of either sample.

(2) Samples of soil were washed with distilled water twice weekly and allowed to dry out at 30–55° between the washings. After 44 days the total N content of the series changed from 0·210 (S.E. 0·001) to 0·197 (S.E. 0·001) %, and 0·004 % of N was recovered in the leachings.

(3) Four samples of fresh soil were packed in glass cylinders and two of them were aerated by an upward current of air for 8 hr. daily over a period of 4 weeks. Each evening both the aerated and the unaerated soils were moistened with

ammonia-free water in amounts just sufficient to ensure that no water ran through the cylinders. On a "dry" basis, the change in total N content was from 0·129 to 0·128 and 0·123 % with the aerated soils, and from 0·129 to 0·128 and 0·126 % with the unaerated soils.

(4) For several reasons, it appeared possible that aerobic losses of N from recently cleared soil in tropical countries might be due to some effect of ultra-violet light. Although both a loss and a gain of N were found in the course of some preliminary experiments in which soil was exposed to a mercury vapour lamp [Corbet, 1935, 1], further work showed that it was rare for an alteration in the total N content of soil to be effected by such a method. We were unable to prove, in fact, that any change was the certain outcome of the action of ultra-violet light, other than the photochemical formation of small amounts of nitrite. Dhar [1935, 1] has reported N fixation by exposure to light of mixtures of cane sugar and sterilized soil in quartz vessels.

Losses due to chemical changes. It has been suggested frequently [e.g. Dhar, 1935, 2], that the chemical disruption of any ammonium nitrite formed in soil may be responsible for the release of N_2, but the studies of Fowler & Kotwal [1924] have shown that losses of N by this means are probably negligible. Jenkins [1933] also considered that there was no experimental evidence to support the view that denitrification results from the chemical decomposition of ammonium nitrite under natural conditions. No N losses occurred when mixtures of sterilized soil (5·0 g.) and aqueous solutions of ammonium sulphate or potassium nitrite (100·0 ml.), contained in a series of flasks, were aerated continuously for 7 days under conditions of sterility, although large losses followed the aeration of similar unsterilized mixtures (Table 1). In these

Table 1. *N losses from soil with added ammonium sulphate and potassium nitrite, during aeration, under sterile and non-sterile conditions*

The figures refer to mg. N contained in the flasks and each is the mean of four results.

(*a*) Under conditions of sterility

Before aeration				After aeration			
Kjeldahl-N	Added NH_3-N	Added NO_2-N	Total N	Kjeldahl-N	NH_3-N in liquid	NO_2-N in liquid	Total N
10·3	11·6	8·6	30·5	10·5	11·2	8·6	30·3

On 4 flasks: Loss of N 0·9
N liberated as NH_3 0·8
Difference 0·1

(*b*) Under non-sterile conditions

Before aeration				After aeration				
Kjeldahl-N	Added NH_3-N	Added NO_2-N	Total N	Kjeldahl N	NH_3-N in liquid	NO_2-N in liquid	NO_3-N in liquid	Total N
8·0	12·6	8·6	29·2	11·6	8·0	4·6	0·0	24·2

On 4 flasks: Loss of N 19·9
N liberated as NH_3 4·3
N presumed lost as gas 15·6

The difference between the NO_2-N content of the 4 flasks before and after aeration was 15·8 mg.

experiments the *p*H value of the supernatant liquid was 7·1–7·2 at the beginning of the experiment; tests showed that sterile conditions were maintained throughout the experiment with sterilized mixtures. The air entering the system was

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ammonia-free (passage through sulphuric acid) and the ammonia liberated from the flasks and carried in the air current was collected and estimated.

Losses due to biological changes. It has been pointed out in the two preceding papers [Corbet & Wooldridge, 1940; Wooldridge & Corbet, 1940], that, in sewage and similar biological systems, the presence of organic matter readily available for the energy needs and growth requirements of heterotrophic micro-organisms leads to synthetic changes accompanied by a proportionate immobilization of ammonia-N. In general, it is only when this process of protein synthesis is completed that nitrification of ammonia-N, if any remains, sets in. In the absence of ammonia-N, however, the conversion of readily available organic matter into more complex forms containing N can be effected at the expense of any nitrite or nitrate present. It was shown that such "organization" of nitrite- or nitrate-N occurs concurrently with losses of N_2, which may be considerable.

By analogy, the necessary conditions for N losses to occur in soil would appear to be the presence of

(1) the necessary micro-organisms or their appropriate enzymes,
(2) organic compounds suitable for synthesis and energy purposes, and
(3) nitrite and/or nitrate as the most readily available N source.

From the physical and chemical experiments already described, the indications are that the presence of micro-organisms is essential for N losses to occur from soil and that their appropriate enzymes are responsible for this loss.

It was found with fresh samples of two different types of soil that, while the micro-flora present effected the conversion of the ammonia-N in sterilized Reader's medium (25·0 ml.) into organic form without N loss, the substitution of nitrate for the ammonia-N was followed by significant losses (Table 2).

Table 2. *Losses of N from a glucose medium containing ammonium sulphate or potassium nitrate and inoculated with soil extract*

The flasks were incubated at 34° for about 5 weeks.
The figures refer to mg. N in the flasks and each in the mean of 4 experiments.

	Initial N content	Final N content	Significance of difference between initial and final N values		
			n	t	P
		(*a*) With $(NH_4)_2SO_4$			
Forest soil (Sussex)	11·3	11·2	6	1·55	Between 0·2 and 0·1
Calcareous soil (Bucks)	11·3	11·3	6	—	—
		(*b*) With KNO_3			
Forest soil (Sussex)	7·6	5·5	6	3·12	Between 0·02 and 0·01
Calcareous soil (Bucks)	7·6	7·1	6	2·74	Between 0·05 and 0·02

The organic matter in soil in a state of biotic equilibrium consists essentially of ligno-proteins [Norman, 1937], so that there is little or no organic matter readily available for bacterial synthesis: the addition of nitrite or nitrate is not, therefore, accompanied by N losses. The experiments set out in Table 3 show no appreciable N losses when a garden soil (5·0 g.) was aerated continuously for 21 days with added solutions of nitrite or nitrate (100·0 ml.). On the other hand in a similar experiment, when a "special" soil of high organic matter content[1]

[1] A "special" soil was prepared by aeration of 250 g. of a similar soil to that used in the experiment detailed in Table 3, in the presence of water, with 25 g. glucose added in 5 biweekly instalments. The supernatant liquid contained no glucose after 2½ weeks when the solid was separated, dried and sieved, and inoculated with fresh soil.

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was aerated for 27 days in the presence of added inorganic nitrogenous salts no N loss occurred in the flasks with additional ammonium sulphate, but significant losses accompanied the aeration of the soil samples with additional nitrite or nitrate (Table 4). In the experiments of Tables 3 and 4 no ammonia-N was liberated during the aeration periods.

It will be observed that the carbon/nitrogen ratios are different in the experiments of Tables 3 and 4, but from the preceding papers it will be evident that the C/N ratio is of itself not the factor controlling the N losses, although, in a general way this ratio reflects the proportions of biologically active C and N compounds present. A high ratio in soil probably betokens the presence of a large amount of easily decomposable organic matter of low N content, although such a state of affairs does not normally persist.

Table 3. *N losses during aeration of a normal soil, in presence of added potassium nitrite or potassium nitrate*

The figures refer to mg. N contained in the flasks, and each is the mean of results from duplicate flasks, except those in the second row of "after aeration" results in (A) which refer to a single experiment. (A) refers to the experiment in which N was added in the form of potassium nitrite and (B) to the experiment with added potassium nitrate.

	Before aeration					After aeration					
	NH_3-N	NO_2-N	NO_3-N	Organic N	Total N	NH_3-N	NO_2-N	NO_3-N	Organic N	Total N	Loss of N
(A)	0·0	0·0	0·0	5·6	5·6	0·0	0·0	0·0	5·6	5·6	0·0
	0·0	2·7	0·0	5·6	8·3	0·0	2·6	0·1	5·6	8·3	0·0
	0·0	5·4	0·0	5·6	11·0	0·0	2·5	2·3	6·2	11·0	0·0
	0·0	10·8	0·0	5·6	16·4	0·0	9·8	0·1	6·1	16·0	0·4
(B)	0·0	0·0	0·0	5·6	5·6	0·0	0·0	0·0	5·7	5·7	0·1 (gain)
	0·0	0·0	2·8	5·6	8·4	0·0	0·0	2·5	5·9	8·4	0·0
	0·0	0·0	5·5	5·6	11·1	0·1	0·0	4·2	6·0	10·3	0·8
	0·0	0·0	11·0	5·6	16·6	0·0	0·0	10·5	5·8	16·3	0·3

C/N ratio of soil before aeration in experiments (A) and (B) = 10·6.

Table 4. *N losses during aeration of a "special" soil, in presence of added inorganic nitrogenous salts*

The figures refer to mg. N contained in the flasks and each is the mean of results from duplicate flasks.

N compound added	Before aeration					After aeration					
	NH_3-N	NO_2-N	NO_3-N	Organic N	Total N	NH_3-N	NO_2-N	NO_3-N	Organic N	Total N	Loss of N
No addition	0·0	0·0	0·0	9·9	9·9	0·0	0·0	0·0	10·1	10·1	0·2 (gain)
$(NH_4)_2SO_4$	3·2	0·0	0·0	9·9	13·1	0·6	0·0	0·0	12·7	13·3	0·2 (gain)
KNO_2	0·0	3·4	0·0	9·9	13·3	0·0	0·0	0·0	10·6	10·6	2·7
KNO_3	0·0	0·0	3·3	9·9	13·2	0·3	0·0	0·0	10·6	10·9	2·3

C/N ratio of soil before aeration in the above experiments = 13·9.

It is possible that such conditions are found in soils in the equatorial belt which are suddenly bereft of forest shade and left exposed to the sun and rain. If, as is claimed, there exists in tropical and subtropical countries either a photochemical [Sarkaria & Fazal-ud-Din, 1933; Dhar & Mukherji, 1935] or a physico-chemical [de Rossi, 1933; 1935] mechanism able to convert organic N into nitrite N and leaving a soil residue of high C/N with excess carbonaceous matter in a form readily available to the micro-flora, the necessary conditions for N liberation would be fulfilled. The process of N impoverishment would be continuous, the nitrite produced during the day being decomposed biologically

during the night with N liberation, until checked by the establishment of vegetation.

We are strongly of the opinion that research into this important problem of N losses by biochemical erosion in soils should be continued by workers in tropical countries and we have put forward our own views at this juncture as we are unable to pursue the matter further.

The analytical procedure followed in this work was the same as that detailed previously by Corbet [1935, 2]. The total N in soil was estimated by Bal's modification of the Gunning-Kjeldahl method, using selenium as a catalyst [Ashton, 1936; 1937]. Removal of nitrite, when present in measurable amount, was found to be a necessary prelude to estimation of the total N, although small amounts of nitrate were "fixed" by the use of reduced iron (Schering-Kahlbaum) as recommended by Ashton.

SUMMARY

It is shown that a biological mechanism exists in soils which is able to effect aerobic losses of N_2 in the presence of

(1) a supply of carbonaceous material suitable for the synthetic and energy requirements of microbial growth, and

(2) nitrite and/or nitrate as the most readily available source of N.

The investigation reported in this and the two preceding papers was carried out as part of the programme of research of the Water Pollution Research Board of the Department of Scientific and Industrial Research, and the results are published by permission of the Department. We wish to express our indebtedness to Prof. J. C. Drummond for his continued interest.

Our thanks are due to the Borough Engineer of Finchley for the regular supplies of sewage used in this work and to Dr W. B. Haines for a supply of soil samples from the Malay Peninsula.

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126. DETERMINATION OF FUMARATE AND MALATE IN ANIMAL TISSUES

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(*Received 27 May 1940*)

Previous workers [see Straub, 1936; Jacobsohn *et al.*, 1932] have determined *l*(−)-malate in animal tissues by the polarimetric method which is based on the high rotation of the uranyl and molybdenum compounds of *l*(−)-malate. Since fumarate forms a well-defined equilibrium with *l*(−)-malate in animal tissues its concentration can be calculated when the concentration of malate is known. The polarimetric method, however, gives only approximate results since the uranyl and molybdenum compounds of other hydroxy-acids occurring in tissues, such as lactate [see Weil-Malherbe, 1937], *iso*citrate [Martius, 1938], phosphoglycerate [Green *et al.* 1937] and α-hydroxyglutarate [Weil-Malherbe, 1937] also show high rotations. The method moreover requires relatively large quantities of malate, 10 mg. in 20 ml. giving a reading of 0·5° in a 2 dm. tube.

In this paper a new method for the determination of *l*(−)-malate and fumarate is described. Fumarate is reduced to succinate in the presence of zinc and phosphoric acid and the succinate formed is determined manometrically with succinic dehydrogenase. The concentration of malate is calculated from the equilibrium constant. This method is more specific than the polarimetric one and it can be applied to quantities of fumarate from 0·05 mg. upwards.

Reagents

1. Metaphosphoric acid, 5% aqueous solution.
2. Zinc filings, 20–30 mesh.
3. $CuSO_4, 5H_2O$, 20% aqueous solution.
4. 10 *M* phosphoric acid solution; 100 ml. phosphoric acid B.P., sp. gr. 1·75, are made up with water to 158 ml.
5. Reagents and apparatus used for the manometric determination of succinic acid [see Krebs, 1937].

Procedure

The following procedure was found to effect the complete conversion of fumarate into succinate.

The tissue suspension, or similar material, is deproteinized at 40° by the addition of 1/5th of its volume of 5% metaphosphoric acid and filtered. An aliquot of the filtrate is transferred to a Kutscher-Steudel extractor, or a measuring cylinder, and 0·5 g. zinc filings, 2·3 ml. phosphoric acid and 0·25 ml. $CuSO_4$ solution for 10 ml. aliquot are added. After 60 min., when the greater part of the Zn has been decomposed, succinic acid is extracted with ether (without further addition of acid) and determined as previously described [see Krebs, 1937; Elsden, 1938; Cohen, 1939].

[1] Fellow of the Rockefeller Foundation.

Recoveries from pure solutions are shown in Table 1. They are within $\pm 5\%$ of the added fumarate. Recoveries from tissue suspensions in which fumarate and malate were in enzymic equilibrium depend on the temperature (Table 2) and on other factors (Table 3). The temperature effect is somewhat smaller than that reported by Jacobsohn [1934], but of the same order of magnitude.

Table 1. *Recovery of fumarate from pure solutions*

Amount of fumarate added (ml. 0·01 *M*)	μl. O_2 absorbed in presence of succinic dehydrogenase	μl. O_2 calc.	Yield (%)
4	442	448	98·6
2	217	224	97·0
1	116	112	103·3
0·5	57·6	56	103·0
0·25	29·4	28	105·0
4	456	448	102·0
4	439	448	98·1
4	486	448	95·6

Table 2. *Recovery of fumarate and l(−)malate added to muscle extract containing fumarase*

10 ml. 0·01 *M* fumarate or *l*(−)malate equivalent to 1120 μl. O_2; 5 ml. 0·1 *M* phosphate buffer *p*H 7·4, 5 ml. muscle extract, made by extracting 1 g. pigeon breast muscle with 50 ml. H_2O; the solutions were kept at different temperatures until the equilibrium was established (30–90 min.).

Temp. ° C.	Substrate used	O_2 absorbed in fumarate determination μl.	Ratio fumarate or malate added / fumarate recovered: Observed	Ratio fumarate or malate added / fumarate recovered: Average	Ratio *l*(−)malate / fumarate: Average
50	Fumarate	302	3·71		
50	,,	309	3·62		
50	,,	306	3·67	3·65	2·65
50	*l*(−)Malate	312	3·59		
50	,,	296	3·68		
40	Fumarate	263	4·25		
40	,,	266	4·21		
40	,,	271	4·13		
40	,,	264	4·24	4·17	3·17*
40	*l*(−)Malate	274	4·09		
40	,,	274	4·09		
30	Fumarate	253	4·42		
30	,,	248	4·50		
30	,,	249	4·49	4·54	3·54
30	,,	245	4·56		
30	,,	237	4·72		
20	Fumarate	195	5·74		
20	,,	196·5	5·70		
20	,,	205	5·46	5·57	4·57
20	,,	196	5·71		
20	*l*(−)Malate	214	5·24		

* In an earlier paper the figure 3·8 was given. This value was obtained at a time when the yield of fumarate was incomplete (about 90%), owing to the use of an excess of acid during the reduction of fumarate.

The ratio $\frac{\text{fumarate or malate added}}{\text{fumarate recovered}}$ (column 5) is the factor by which the fumarate or malate found in the presence of fumarase is to be multiplied in order to obtain the sum of fumarate and malate present. The value of this figure is not appreciably

Table 3. *Effect of tungstate on the equilibrium between fumarate and* l(−)*malate*

Conditions as in Table 2, 40°.

Concentration of Na tungstate %	Fumarate added (in terms of μl. O_2)	Fumarate recovered (in terms of μl. O_2)	Ratio $\frac{\text{fumarate added}}{\text{fumarate recovered}}$
0	1120	269	4·17
0·5	1120	235	4·76
2·0	1120	111	10·1

affected by *p*H within 6·8 and 7·8, or by the ions of the tissue and the saline media.

Specificity. Malate, tartrate, oxaloacetate, aspartate, glutamate, citrate, aconitate do not react to form succinate under the above conditions. Maleic acid behaves like fumaric acid, but since it is not present in biological material, it does not need to be considered. If the solution contains succinate, it must be determined separately in an aliquot before the treatment with Zn and acid, and the value obtained must be deducted from the succinate found in the fumarate determination.

Notes

1. *Deproteinization.* Tungstic acid (Folin-Wu) is not suitable as a deproteinizing agent. It was found that addition of sodium tungstate to the tissue suspension rapidly shifts the equilibrium fumarate ⇌ malate in favour of malate (Table 3). This is due to the formation of sodium tungstimalate $(NaC_4H_4O_5)_2WO_2$ [see Gernez, 1890; Henderson *et al.*, 1899]. Deproteinizing agents which are ether-soluble, e.g. trichloroacetic acid, are also to be avoided, as they interfere with the determination of succinic acid.

When fumarase is present, deproteinization must be carried out under defined conditions, since the equilibrium between fumarate and malate depends on the temperature (see Table 2) and in general it will be advisable to deproteinize at the temperature of the experiment in which the fumarate was formed.

There seems to be no appreciable loss of fumarate by adsorption on the precipitated protein.

2. *Stability of metaphosphoric acid solution.* HPO_3 in aqueous solution is gradually converted into H_3PO_4. At 0° the conversion is complete in 150 days, at 14° in 30 days, at 31° in 5 days, at 61° in 4½ hr. and at 95° in less than 1 hr. [Sabatier, 1888]. It follows that the solution must be prepared without heating and that it can be stored for a short time in the cold.

3. *Zinc preparations.* The commercial zinc preparations vary greatly in their readiness to react with phosphoric acid. These differences are largely eliminated by the addition of $CuSO_4$.

4. *Concentration of acid.* The yields of succinate are incomplete when the acidity during the reduction is high, for instance, when an excess of sulphuric acid is used. H_3PO_4 was chosen because it is weaker than H_2SO_4. A large excess of phosphoric acid is to be avoided.

5. *Frothing during the reduction of fumarate.* If excessive frothing occurs during the evolution of H_2, a drop of capryl alcohol may be added to the suspension. The alcohol later passes into the ethereal extract and is removed by evaporation on the steam bath together with the ether.

6. *Ether.* The ether used must be free from peroxide. It is kept over sodium and distilled freshly before use.

7. *Extraction of succinic acid.* The main cause of low yields is incomplete extraction of succinic acid and special attention must therefore be paid to the efficiency of the extractor. We use continuous extractors of the Kutscher-Steudel type, of the dimensions shown in Fig. 1. Each unit is provided with ground glass joint No. A 29 and with a set of 3 internal funnels of varying diameter (6 mm., 13 mm. and 16 mm.). The wider funnels are used when the volume of the aqueous solution is relatively small. A wide funnel increases the height of the aqueous layer, and thereby the efficiency of the extraction. If the lower end of the funnel is clean-cut, the ether bubbles passing through the aqueous solution are very small and no advantage is afforded by the use of sintered glass in the funnel bottom.

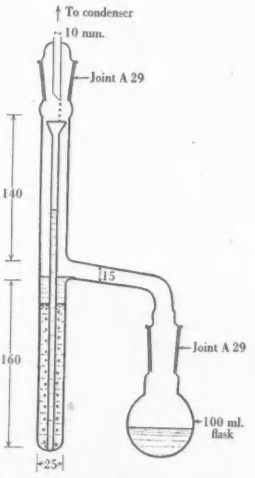

Fig. 1. *Extractor.* The dimensions are given in mm. and refer to internal diameters.

Since the rate of extraction depends on many factors, it is difficult to define standard conditions which ensure complete extraction. It is therefore advisable to test the extractor with known amounts of succinate. The following data may serve to give an approximate estimate of the rate of extraction. In an experiment where the volume of the aqueous phase was 50 ml. and the amount of ether passing through the solution about 16 ml. per min., 56 % of the succinic acid was extracted in 30 min., 73 % in 60 min., 96·5 % in 120 min., 99·3 % in 180 min. and 100 % in 240 min.

In the routine procedure the efficiency of the extraction can be tested by means of a dye of a suitable partition coefficient, the extraction of which can be easily followed with the eye. We have examined[1] a number of such "extraction indicators" and found that their rates of extraction fall in the following order:

cresol red > phenol red > rosolic acid > litmus.

Since the dye must not interfere with the later adjustment of *p*H of the extracted material, dyes were used which can also serve as *p*H indicators in the neutral range. Rosolic acid (1 ml. 0·1 % solution) was found to be the best indicator for the extraction of succinic acid. Its extraction is somewhat slower than that of succinic acid and the almost complete extraction of the indicator therefore ensures complete extraction of succinic acid.

When the extraction is complete, 1 ml. 0·1 *M* Na_2HPO_4 solution is added to the ether and the ether is evaporated on the steam bath. To remove the last traces of the ether, the flask is left on the bath until about 50 % of the aqueous solution has been evaporated. The residue is then transferred, with a 1 ml. pipette, to a 5 or 10 ml. measuring cylinder, neutralized to approximately *p*H 7·0 (rosolic acid or phenol red) and made up to a known volume (1–3 ml.) with the washings from the extraction flasks.

8. *Succinic dehydrogenase.* Succinic dehydrogenase is prepared by washing minced pigeon breast muscle or mammalian heart three times with water. For

[1] Preliminary experiments on the use of extraction indicators were carried out in this department by Dr P. P. Cohen.

storage, the washed muscle is frozen in the freezing box of the refrigerator; the preparation keeps for several weeks in the frozen form (P. P. Cohen, personal communication).

SUMMARY

A method for the determination of fumarate and *l*(−)malate is described. Fumarate is reduced to succinate in the presence of zinc and phosphoric acid and the succinate formed is determined manometrically with succinic dehydrogenase. The concentration of *l*(−)malate is calculated from the equilibrium constant. At *p*H 7·4 the ratio *l*(−)malate/fumarate was found to be 2·65 at 50°, 3·17 at 40°, 3·54 at 30° and 4·57 at 20°.

This work was aided by grants from the Rockefeller Foundation and from the Medical Research Council.

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127. THE OXIDATION OF PYRUVATE BY HEART MUSCLE

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(*Received 1 June 1940*)

Braun-Menendez *et al.* [1939], working with heart-lung and isolated heart preparations, have shown that pyruvate can be utilized by the mammalian heart. Minced heart muscle, as will be seen later, oxidizes pyruvate at about the same rate as the intact heart, and it was used in the present work for a study of the mechanism of the oxidation of pyruvate. The object of the investigation was to determine whether oxidation occurs by way of the citric acid cycle as in pigeon breast muscle [Krebs & Eggleston, 1940].

The main evidence for the occurrence of the citric acid cycle in pigeon breast muscle is as follows:

(1) The oxidation of pyruvate is inhibited by malonate.

(2) The malonate inhibition is partially overcome by the addition of fumarate, and the extra amount of pyruvate used in these conditions is in the proportion of 1 mol. of pyruvate to each mol. of added fumarate. In the presence of malonate succinate is formed in amounts equivalent to the fumarate added, the reaction being

$$\text{fumarate} + \text{pyruvate} + 2O_2 \rightarrow \text{succinate} + 3CO_2 + H_2O. \qquad (1)$$

(3) Under certain conditions fumarate and pyruvate can be shown to form citrate and α-ketoglutarate.

The general method of investigation in this work was to examine if these same reactions can be demonstrated in heart muscle, and it will be shown that the process of pyruvate oxidation in mammalian heart muscle is fundamentally the same as in avian skeletal muscle.

Methods

In general the procedure followed closely that described by Krebs & Eggleston [1940], and the same methods were used for the various estimations.

The tissue used was sheep's heart, obtained from the abattoir. As soon as possible after killing the animal the heart was removed, cooled to 0° and maintained at this temperature until the experiment was begun. The rapid cooling after death was found to be very important, and if not carried out within a few min. the rates of O_2 consumption and of pyruvate utilization were found to be greatly reduced. The muscle of the left ventricle was minced in the Latapie mill, and suspended in ice-cold phosphate-saline. Except where otherwise stated a suspension consisting of 1 g. of muscle and 7·5 ml. saline were used; 3 ml. of this were measured into a Warburg cup, and the volume made up to 4 ml. with added substrates, inhibitors and distilled water. The respiration was measured for periods varying from 30 min. to 2 hr. at 40° in the presence of various substrates. The usage of the added substrates was measured, and also the formation of succinate, α-ketoglutarate and citrate under various conditions.

In expressing the results the amounts of all substances are expressed as μl. of gas, 1 millimol being taken as equivalent to 22,400 μl.

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1. *Oxidative removal of pyruvate*

Pyruvate usage. Pyruvate added to suspensions of heart muscle in the presence of O_2 is readily oxidized. The amounts used and also the time course of the pyruvate usage are shown in Table 1. In the first experiment quoted there

Table 1. *Pyruvate utilization and O_2 consumption in heart muscle*

(4 ml. suspension)

Heart muscle mg.	Initial conc. of pyruvate *M*	Duration of experiment min.	Pyruvate used μl.	O_2 used μl.	$\frac{O_2 \text{ used}}{\text{Pyruvate}}$
375	0·02	30	293	559	1·91
		60	573	1134	1·97
		120	1113	2569	2·31
353	0·02	120	882	1868	2·11
353	0·02	120	1333	2461	1·84
353	0·02	125	884	1979	2·24
353	0·02	120	944	2000	2·12

is no falling off in the O_2 consumption or in the pyruvate usage for the 2 hr. period. In most experiments however there is a progressive decrease in the oxidative processes as measured by the O_2 consumption (Table 2), so that the

Table 2. *Effect of pyruvate on the respiration of heart*

(4 ml. suspension)

Heart muscle mg.	Initial conc. of pyruvate *M*	O_2 consumption (μl.) 30 min.	60 min.	120 min.
375	0	456	711	1001
	0·02	603	1215	2579
353	0	500	770	880
	0·02	650	1260	1870
353	0	430	701	1059
	0·02	630	1249	2461
353	0	396	574	640
	0·02	700	1367	2100
400	0	300	500	659
	0·02	653	1273	2141

initial rate of pyruvate usage is probably greater than the values shown, which are the average rates over the whole 2 hr. period. At a concentration of pyruvate of 0·02 *M* the amounts used vary from 474 to 744 mg. pyruvic acid/100 g. heart muscle/hr. Similar figures were reported for the intact heart by Braun-Menendez *et al.* [1939], who obtained values varying from a maximum of 870 mg./100 g./hr. to very low values depending among other factors on the concentration of pyruvate. The fact that minced heart uses pyruvate at about the same rate as the intact heart may be taken as evidence that the processes occurring in the minced tissue are of a similar nature to those in the intact beating heart.

The values for pyruvate given here include the total pyruvate which disappeared during the experiment. Anaerobic controls showed that while there was a definite anaerobic disappearance of pyruvate also taking place this did not amount to more than 20% of the total pyruvate used.

O_2 usage. The figures given in Tables 1 and 2 show the rate of O_2 consumption. In the presence of pyruvate the Q_{O_2} varies from 16 to 20, which is equivalent to a usage of 320 to 400 ml./100 g. fresh tissue/hr. The higher values are approaching

the values recorded for the intact beating dog's heart doing the maximal amount of work. Evans *et al.* [1935], from estimation of the O_2 content of the coronary arterial and venous blood in the heart-lung preparation, found values of 221 ml./100 g./hr. Higher values have been found in other experiments. With varying conditions of activity Evans [1912] found O_2 consumptions of 250 to 500 ml./100 g./hr., and other results fall within this range [Starling & Evans, 1914; Cruickshanks & Startup, 1934; Gremels, 1933; Cohn & Steele, 1935]. Few corresponding data are available for the *in vitro* respiration of the heart. Tsuneyoshi [1927] reported a Q_{O_2} of about 20 for the rabbit's heart and for the same tissue Stare & Baumann [1939] found a Q_{O_2} of 12–17. It is seen again that the oxidative processes in minced heart and in active intact heart are of the same order of magnitude.

The complete oxidation of 1 mol. of pyruvate requires $2\frac{1}{2}$ mol. of O_2. The figures given show that this ratio of O_2 to pyruvate was approached but never attained. This is possibly explained by the simultaneous anaerobic disappearance of a certain amount of pyruvate, or possibly by incomplete oxidation of the pyruvate. As will be shown later this is known definitely to occur under certain conditions.

The addition of pyruvate in a concentration of 0·02*M* to heart muscle has a marked effect in increasing the respiration (Table 2). The effect as noted by Krebs & Eggleston in pigeon breast muscle is more marked in the later stages of the experiment. In the case of heart muscle the magnitude of the effect of pyruvate is much greater and results in an increase of about 150 % in the 2 hr. period as compared with an increase of 30 % in pigeon breast muscle. The increase in O_2 consumption on addition of pyruvate is not however sufficient to account for the pyruvate which disappears, so that the added pyruvate is presumably oxidized in preference to other substances already present in the heart.

Effect of the concentration of pyruvate. The increase in O_2 consumption on the addition of pyruvate depends on the concentration of pyruvate used. Maximal respiration and pyruvate usage over 2 hr. periods were obtained with about 0·02*M* initial concentration. Experiments carried out for shorter periods showed

Table 3. *Effect of concentration of pyruvate on the O_2 consumption and pyruvate utilization*

(400 mg. heart muscle in 4 ml.)

Initial conc. of pyruvate *M*	O_2 consumption (μl.) 30 min.	O_2 consumption (μl.) 60 min.	Pyruvate used μl. 60 min.	O_2 used / Pyruvate used
0	547	1006	—	—
0·01	628	1273	585	2·18
0·02	588	1157	639	1·81
0·03	432	772	635	1·21
0·04	331	492	400	1·22
0·05	261	338	—	—
0	523	913	—	—
0·0075	624	1278	—	—
0·025	559	1189	—	—
0·04	516	1084	—	—
0·0075	535	1145	438	2·61
0·015	562	1118	524	2·13
0·03	598	1186	726	1·63
0	414	514	—	—
0·0075	676	1295	—	—
0·025	521	847	—	—

that lower concentrations (0·0075 *M*) usually caused a slightly higher rate of respiration, but that higher concentrations always caused a diminution of O_2 consumption, which in the case of the highest concentration tested, 0·05 *M*, reached 70 % inhibition. This is illustrated in Table 3, which also includes some figures for the pyruvate disappearance with different initial concentrations of pyruvate. While the amount of pyruvate used does not always diminish with the rising concentration of pyruvate, the ratio of O_2 used to pyruvate does so in every case, so that only with the lowest concentrations is the pyruvate which disappears oxidized completely. This inhibition of the oxidative processes by large amounts of pyruvate is used later to demonstrate some of the intermediate products of pyruvate metabolism.

2. *Malonate inhibition*

Malonate inhibits both the O_2 consumption and the pyruvate usage of heart muscle. Table 4 gives the figures for pyruvate utilization. At the highest concentration of malonate used, 0·025 *M*, there is an inhibition of pyruvate usage up

Table 4. *Effect of malonate on the usage of pyruvate*

(353 mg. heart muscle in 4 ml.)

Conc. of malonate *M*	Initial conc. of pyruvate *M*	Pyruvate used in 120 min. μl.
0	0·0075	882
0·001	0·0075	697
0	0·02	944
0·01	0·02	254
0	0·02	1333
0·025	0·02	238
0	0·0075	504
0·025	0·0075	206

Table 5. *Effect of malonate on the O_2 consumption of heart muscle*

(353 mg. heart muscle in 4 ml.)

Conc. of malonate *M*	Initial conc. of pyruvate *M*	O_2 consumption (μl.)		
		30 min.	60 min.	120 min.
0	0	456	711	1001
0·001	0	290	485	570
0	0·02	690	1260	1868
0·001	0·02	383	755	979
0	0	430	701	1059
0·025	0	89	117	141
0	0·02	630	1249	2461
0·025	0·02	133	180	208
0	0·0075	601	1020	1151
0·025	0·0075	59	85	109
0	0·02	572	1138	2000
0·01	0·02	183	267	351
0	0·02	492	758	908
0·01	0·02	69	98	128

to about 80 %. Since a part of the residual pyruvate disappearance is probably due to an anaerobic reaction, the inhibition of the oxidative removal of pyruvate must be regarded as higher than this value and possibly nearly complete.

Table 5 shows the very marked inhibition of O_2 consumption by various concentrations of malonate. The O_2 consumption is also greatly reduced by malonate when no pyruvate is added.

Table 6. *Effect of fumarate on the removal of pyruvate in the presence of malonate*

(4 ml. suspension)

Heart muscle mg.	Initial conc. of pyruvate *M*	Malonate *M*	Fumarate added µl.	Pyruvate used µl.	Extra pyruvate µl.
353	0·0075	0·001	0	697	—
353	0·0075	0·001	448	1027	330
353	0·02	0·025	0	238	—
353	0·02	0·025	448	683	445
353	0·0075	0·025	0	429	—
353	0·0075	0·025	56	536	107
353	0·0075	0·025	112	560	131
353	0·02	0·01	0	254	—
353	0·02	0·01	224	524	270
353	0·02	0·01	448	574	320
353	0·0125	0·025	0	65	—
353	0·0125	0·025	224	230	165
353	0·0125	0·025	448	388	323
400	0·01	0·025	0	109	—
400	0·01	0·025	56	177	68
400	0·01	0·025	112	211	102
400	0·01	0·025	224	330	221
400	0·01	0·025	448	586	477
353	0·02	0·001	0	312	—
353	0·02	0·001	56	545	233
353	0·02	0·001	112	590	278

Table 7. *Effect of fumarate on O_2 consumption in the presence of malonate*

(4 ml. suspension)

Heart muscle mg.	Duration of exp. min.	Initial pyruvate conc. *M*	Malonate conc. *M*	Fumarate added µl.	O_2 used µl.	Extra O_2 µl.
353	120	0·0075	0·001	0	979	—
353	120	0·0075	0·001	448	2325	1346
353	120	0·02	0·025	0	208	—
353	120	0·02	0·025	448	1098	890
353	120	0·0075	0·025	0	383	—
353	120	0·0075	0·025	56	575	192
353	120	0·0075	0·025	112	716	333
353	120	0·0075	0·025	224	999	616
353	120	0·0075	0·025	448	1474	1091
353	120	0·0125	0·025	0	194	—
353	120	0·0125	0·025	224	625	431
353	120	0·0125	0·025	448	902	708
353	120	0·02	0·01	0	210	—
353	120	0·02	0·01	56	452	242
353	120	0·02	0·01	112	607	397
353	120	0·02	0·01	224	789	579
353	120	0·02	0·01	448	941	731
400	90	0·01	0·025	0	187	—
400	90	0·01	0·025	56	312	125
400	90	0·01	0·025	112	434	247
400	90	0·01	0·025	224	603	416
400	90	0·01	0·025	448	1052	865

Effect of fumarate. The effect of fumarate in removing the malonate inhibition of both pyruvate usage and O_2 consumption is shown in Tables 6 and 7. The extra amount of pyruvate which disappears in the presence of malonate is roughly proportional to the amount of fumarate added, the relation being 1 mol. of pyruvate to 1 mol. of added fumarate. These results are explained by the hypothesis of the citric acid cycle, and so far no alternative explanation is available. In the case of lower concentrations of malonate the fumarate may produce a catalytic action, as illustrated in the last experiment quoted in Table 6, where in presence of a concentration of 0·001 *M* malonate the addition of 56 and 112 μl. of fumarate led to the removal of 233 and 278 μl. of pyruvate. In this case the malonate inhibition, owing to its competitive nature, is overcome by the succinate formed during oxidation of the pyruvate.

The O_2 consumption is also increased by the addition of fumarate and the increase bears a fairly definite relationship to the amount of fumarate added. According to equation (1) the formation of succinate from fumarate and pyruvate in the presence of malonate requires 2 mol. of O_2 for each mol. of fumarate and from Table 7 it is seen that the extra O_2 used is of this order.

Effect of citrate and α-ketoglutarate. Since citrate and α-ketoglutarate, according to the citric acid cycle, are intermediate products in the formation of succinate from pyruvate and fumarate, they should be capable of increasing the respiration in the presence of malonate. With α-ketoglutarate this is certainly true and in some cases the extra O_2 on addition of ketoglutarate is of the order of $\frac{1}{2}$ mol. of O_2 to 1 mol. of ketoglutarate, i.e. the amount necessary to convert the ketoglutarate into succinate (Table 8). In the case of citrate there is some variation

Table 8. *Effect of citrate and α-ketoglutarate on the O_2 consumption in presence of malonate*

(4 ml. suspension)

Heart muscle mg.	Duration of exp. min.	Malonate conc. *M*	Citrate added μl.	α-ketoglutarate added μl.	O_2 used μl.
353	90	0·025	—	—	155
353	90	0·025	448	—	186
353	90	0·025	—	448	232
353	120	0·01	—	—	187
353	120	0·01	448	—	53
353	120	0·01	—	448	226
353	60	0·005	—	—	157
353	60	0·005	448	—	56
353	60	0·00125	—	—	301
353	60	0·00125	448	—	49
353	120	0·01	—	—	316
353	120	0·01	224	—	407
353	120	0·01	448	—	503
353	115	0·005	—	—	249
353	115	0·005	896	—	393
353	115	0·005	—	896	590

in the results, as in some cases the addition of citrate in the presence of malonate increases the respiration, while in others the inhibition of respiration is more marked on the addition of citrate. Somewhat similar observations were made by Stare & Baumann [1939]. Even where there is an increase in respiration this is not so great as that produced by addition of ketoglutarate, although the amount of O_2 required to convert citrate into succinate is twice that required to convert the corresponding amount of α-ketoglutarate.

There are several possible explanations for the citrate effects.

(1) The malonate may cause an inhibition of the citric dehydrogenase as suggested by Stare & Baumann [1939].

(2) The succinic and other dehydrogenases may be inhibited by citrate.

(3) Since the oxidation of citrate is associated with the reduction of oxaloacetate the amounts of this substance present will influence the effect of citrate on the malonate inhibition.

These possibilities are being further investigated.

Formation of succinate. According to equation (1) the fumarate added in the presence of malonate is converted into succinate, the intermediate products of reaction being citrate and α-ketoglutarate. That this occurs in heart muscle is shown by the fact that succinate can be recovered on addition of these substances in the presence of malonate (Table 9). It has already been pointed out that under

Table 9. *Formation of succinate in the presence of malonate*

(4 ml. suspension)

(*a*) From fumarate

Heart muscle mg.	Duration of exp. min.	Initial conc. of pyruvate *M*	Conc. of malonate *M*	Fumarate added μl.	Succinate formed (μl.) Total	Extra
353	140	0·0125	0·01	—	114	—
353	140	0·0125	0·01	224	332	218
353	140	0·0125	0·01	448	402	288
353	120	0·02	0·01	—	60	—
353	120	0·02	0·01	448	290	230
400	60	0·01	0·025	—	104	—
400	60	0·01	0·025	112	180	76
400	60	0·01	0·025	224	254	150
353	140	—	0·01	—	92	—
353	140	—	0·01	224	296	204
400	60	—	0·025	—	96	—
400	60	—	0·025	112	140	44
400	60	—	0·025	224	182	86

(*b*) From citrate and oxaloacetate

Heart muscle mg.	Duration of exp. min.	Substrate and amount added	Conc. of malonate *M*	Succinate formed μl.	Extra succinate μl.
353	120	None	0·01	60	—
353	120	224 μl. citrate	0·01	161	101
353	120	448 μl. citrate	0·01	258	198
353	120	448 μl. oxaloacetate	0·01	167	107
353	120	448 μl. oxaloacetate + 448 pyruvate	0·01	322	262
400	90	672 μl. citrate	0·01	40	—
		1344 μl. citrate	0·01	260	—
		2688 μl. citrate	0·01	232	—

these conditions conversion of citrate into succinate is incomplete, and this is in agreement with the smaller amounts of succinate found on addition of citrate as compared with fumarate. It is also seen that even in the absence of added pyruvate a certain amount of succinate is formed. If it is assumed that also in the absence of added pyruvate succinate is formed by reaction (1), it follows that the heart can form pyruvate from substances already present, and hence that pyruvate is normally an intermediate product in the metabolism of the heart muscle.

3. *Formation of citrate and α-ketoglutarate*

High concentrations of pyruvate have been shown to cause an inhibition of the respiration and an incomplete oxidation of the pyruvate which disappears. This inhibition was used by Krebs & Eggleston [1940] to demonstrate the formation of the intermediate products of reaction, citrate and α-ketoglutarate. Table 10 shows that formation of these substances also takes place in the heart. While small amounts of citrate are formed in the absence of added fumarate the amounts formed are greatly increased by the addition of this substance.

Table 10. *Formation of citrate and α-ketoglutarate*

Heart muscle mg.	Initial conc. of pyruvate *M*	Fumarate added μl.	Ketoglutarate formed μl.	Citrate formed μl.
353	0	—	84	20
353	0·02	—	163	20
353	0·04	—	166	—
353	0·04	448	308	—
353	0·04	—	138	9
353	0·04	448	412	30
400	0·02	—	—	17
400	0·02	224	—	51
400	0·04	—	—	17
400	0·04	224	—	38

4. *Usage of citrate*

Since Breusch [1939] has questioned the usage of citrate by the heart, figures are given to show that citrate added to minced heart disappears (Table 11). The amount of citrate used is of the order of magnitude postulated by the citric acid cycle.

Table 11. *Aerobic and anaerobic usage of citrate*

(4 ml. suspension)

(*a*) Aerobic

Heart muscle mg.	Period of exp. min.	Initial conc. of citrate *M*	Citrate used μl.
400	90	0·0075	288
400	90	0·015	474
400	90	0·03	326
353	90	0·01	269

(*b*) Anaerobic

Heart muscle mg.	Period of exp. min.	Initial conc. of citrate *M*	Initial conc. of oxaloacetate *M*	Change in citrate μl.	Ketoglutarate formed μl.
666	60	0·02	0	− 11	0
666	60	0·02	0·02	−449	782
666	60	0	0·02	+231	340

Citrate can also be used anaerobically in the presence of oxaloacetate in the same way as in pigeon breast muscle [Krebs, 1940]. From the figures in Table 11 it may be concluded that the reaction described by Krebs also occurs in heart muscle

$$\text{Citrate} + \text{oxaloacetate} \rightarrow \alpha\text{-ketoglutarate} + \text{malate} + CO_2.$$

5. *Maintenance of the respiration by substrates other than pyruvate*

Stare & Baumann [1939] have shown that citrate and fumarate can each cause increase in the respiration of the heart. The effect of these and other substances was investigated. Table 12 shows the effects of a number of substances

Table 12. *Rate of respiration by heart muscle in the presence of various substances*

Heart muscle mg.	Substrate added	Conc. of added substrate *M*	O_2 consumption 30 min.	60 min.	120 min.
353	—	—	391	533	590
353	Citrate	0·01	557	824	905
353	α-Ketoglutarate	0·01	747	1493	1945
400	—	—	300	500	659
400	Pyruvate	0·01	653	1273	2141
400	Fumarate	0·01	486	959	1745
400	Citrate	0·01	530	980	1604
400	Succinate	0·01	729	1208	1852
400	Aspartate	0·01	319	560	783
400	Glutamate	0·01	645	1285	1953
400	Fumarate + Pyruvate	0·0025 + 0·01	715	1482	2480
353	—	—	368	441	493
353	Citrate	0·005	423	834	1089
353	Citrate	0·01	543	883	1069
353	Citrate	0·02	623	1078	1440
353	Pyruvate	0·02	570	1175	1860
353	—	—	317	461	—
353	Citrate	0·01	383	864	— (90 min.)
400	—	—	456	821	1213
400	Citrate	0·0075	624	1220	1825
400	Citrate	0·015	558	1116	1676
400	Citrate	0·03	513	989	1415

on the O_2 consumption of the heart. These include most of the substances of the citric acid cycle, and also the related substances aspartate and glutamate. All of the substances tested with the exception of aspartate increase and maintain the respiration of the heart. Citrate would appear to have a smaller effect than the other substances tested, and also the effect of citrate may decrease with increasing concentrations.

Discussion

It has been shown that the main reactions on which the citric acid cycle is based occur in minced heart muscle. These reactions are the malonate inhibition of O_2 consumption and pyruvate usage, the specific effect of fumarate in combining with pyruvate to form succinate in the presence of malonate, the formation of citrate and α-ketoglutarate, and the effect of the substances involved in the citric acid cycle in maintaining respiration.

This view that the citric acid cycle plays a part in the metabolism of heart muscle is supported by the work of Hallman & Simola [1939], who showed that the synthesis of citric acid in heart muscle can occur in the presence of pyruvate and fumarate or oxaloacetate, and also that the citric acid can break down to α-ketoglutarate, succinate, fumarate, malate and oxaloacetate.

Heart muscle also provides an opportunity not given by skeletal muscle of comparing the relative rates of the oxidative processes in minced tissue and in

physiologically active tissue. The pyruvate consumption found is of the same order as that in the intact beating heart, while the O_2 consumption approaches the highest values found for the heart working at maximal effort. While the data from which these comparisons are made are obtained from different species, observations by Van Heyningen [1936] indicate a uniformity in the rate of metabolism in the same tissue in different species irrespective of the size of the animal.

In the intact heart Braun-Menendez *et al.* [1939] found that high concentrations of pyruvate gave rise to extra-systoles and other cardiac irregularities. It is of interest that the concentration of pyruvate (above 0·02 *M*) which produced these effects caused in the minced heart a decrease in the stimulating effect of pyruvate on respiration and an incomplete oxidation of the pyruvate used.

Certain observations made here point to pyruvate as a normal intermediate substance in the metabolism of the heart. These are:

(1) The effect of pyruvate in maintaining the respiration of the heart in the absence of other added substrates.

(2) The inhibition of respiration by malonate even in the absence of added pyruvate.

(3) The ability of fumarate in these conditions to restore partially the respiration and to form succinate.

Summary

1. Minced heart muscle of the sheep used O_2 and pyruvate at about the same rate as the intact working heart of the dog.

2. Both the O_2 consumption and the pyruvate utilization are inhibited by malonate.

3. This inhibition is partially removed by the addition of fumarate. Each mol. of fumarate added causes the removal of one extra mol. of pyruvate, and an extra consumption of 2 to 3 mol. of O_2. Succinate is formed in amounts approximately equivalent to the added fumarate.

4. Addition of pyruvate in concentrations up to 0·02 *M* maintains the respiration of the minced heart. Higher concentrations have an inhibitory effect on O_2 consumption, and the pyruvate which disappears is incompletely oxidized.

5. The inhibitory action of high concentrations of pyruvate results in the appearance of citrate and α-ketoglutarate. The amounts of these formed are increased by the addition of fumarate.

6. Citrate added to minced heart muscle disappears in presence of molecular O_2 and also anaerobically in presence of oxaloacetate.

7. The substances involved in the citric acid cycle, pyruvate, fumarate, succinate, citrate and α-ketoglutarate, are all capable of increasing the respiration of the heart.

8. These observations support the view that pyruvate is oxidized in the heart by way of the citric acid cycle and they cannot be satisfactorily explained by any other hypothesis.

I am indebted to Dr H. A. Krebs for generous facilities provided for carrying out this work, and also for his constant advice and assistance.

Part of the expenses of the work were defrayed by a grant from the Thomas Smythe Hughes Fund for Medical Research from the University of London.

Part of the expenses were also defrayed by grants to Dr Krebs from the Rockefeller Foundation and from the Medical Research Council.

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128. AMMONIA PRODUCTION BY PATHOGENIC BACTERIA

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(*Received 3 May 1940*)

THE discovery of glutamine as a growth factor for *Streptococcus haemolyticus* [McIlwain *et al.* 1939] and the occurrence of hydrolytic and synthetic glutaminase in those animal tissues, kidney and liver [Krebs, 1935] which contain the most active deaminases [Krebs, 1933], suggested that glutamine might play a part in the metabolism of amino-acids by streptococci, possibly as a carrier of labile NH_3. It was considered desirable therefore to examine the deamination of amino-acids by the organism since no interest has been shown in this field, though a soluble protease has been extracted by Stevens & West [1922]. Foster [1921] showed that NH_3 was liberated coincidently with disappearance of NH_2-N during the period of most active growth on glucose-broth or glucose-serum-broth, though subsequently NH_2-N reappeared without disappearance of NH_3. This was assumed to be due to continued proteolysis without further utilization of amino-acids. Out of 101 substrates tested as H-donators to indigo-tetrasulphonate, 71, including the amino-acids, were found by Farrell [1935] to be inactive. The present work reconciles these findings, since it is shown that out of 22 α-amino-acids only arginine yields significant amounts of NH_3 and the reaction is not oxidative but hydrolytic. As the same behaviour was shown by streptococci of diverse habitat and nutritional requirements, the study was extended to staphylococci. Minor differences were shown but arginine was quantitatively the most important source of NH_3, urea, the next best, being not more than half as active.

The observations of Fildes [1934] and Knight [1936] suggested that this restricted metabolic activity might be related to a habitat in association with the products of animal metabolism with the ultimate development of pathogenicity in many of the organisms examined. Comparative data for representative human and animal pathogens of other genera were lacking; early work on amino-acid breakdown was restricted to chemical identification of the products of putrefaction, often by mixed cultures of unidentified organisms, while the recent work of the Cambridge school, under better controlled conditions, has dealt almost entirely with strict anaerobes and *Bacterium coli* (for bibliography see Stephenson [1939]). Experiments on the meningococcus and the diphtheria and typhoid organisms were therefore carried out. The first two showed even more restricted chemical activity than the gram-positive cocci previously examined and the last, though an active NH_3 producer, resembled *Bact. coli*, forming little NH_3 from arginine.

To find how far the breakdown of arginine was a constant character of gram-positive cocci, experiments were carried out with a strain of staphylococcus trained to grow on a synthetic medium with NH_4^+ salts as the main source of N [Gladstone, 1937, 2]. With such a strain it is possible to alter at will any adaptive

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enzymes by controlled variation of the medium with little change in the total growth to complicate interpretation of results. *Bact. typhosum* has also been trained to dispense with a preformed supply of amino-acids [Fildes *et al.* 1933] and the effect of training was also observed in this case.

Technique

(1) *Organisms.* The strains used were:

1. *Str. haemolyticus.* Richards. Group A from Dr L. Colebrook.
2. *Str. haemolyticus.* Kenny. Group B from Dr L. Colebrook.
3. *Str. haemolyticus.* N. Morris. Group B from Dr L. Colebrook.
4. *Str. haemolyticus.* Batty. Group B from Dr L. Colebrook.
5. *Str. viridans.* N.C.T.C. No. 3165.
6. *Str. faecalis.* Freshly isolated by Dr G. P. Gladstone.
7. *Str. lactis.* N.C.T.C. No. 2700.
8. *Staph. aureus.* Strain No. 1 of Gladstone [1937, 2].
9. *Staph. aureus.* Trained from No. 8 [Gladstone, 1937, 2].
10. *N. intracellularis.* N.C.T.C. No. 3375.
11. *Bact. typhosum.* Rawlings. N.C.T.C. No. 160.
12. *Bact. typhosum.* Trained from No. 11 [Fildes *et al.* 1933].
13. *C. diphtheriae.* Mitis strain from Dr A. B. Rosher.

(2) *Media for growth.* The basal medium used for general purposes was, with the addition of glutamine, designed for the growth in bulk of group A strains of haemolytic streptococci in the light of the findings of Fildes & Gladstone [1939] and McIlwain [1939; 1940]. It consisted of:

Bacto-peptone	10 g.	in 250 ml. H_2O
NaCl	5 g.	
KH_2PO_4	4·5 g.	
Na glutamate	1·7 g.	
Cystine	0·05 g.	
N NaOH	28 ml.	
Yeast extract	50 ml.	

The mixture, adjusted to *p*H 7·6, together with various additions usually made after autoclaving, gave 1 l. medium. The organisms were grown in 250 ml. conical flasks, each containing 100 ml. medium final volume. The additions were, per flask, 1 ml. 0·02 *M* glutamine, 2·5 ml. 0·5 *M* glucose (medium A_G), 5 ml. 0·5 *M dl*-lactate (medium A_L), 1 ml. 0·5 *M* arginine (medium A_A or A_{AG} if glucose was present in addition) or, for meningococcus (*Neisseria intracellularis*) only, 3 ml. horse serum (medium A_S). The inoculum was 0·2 ml. of a just visibly turbid suspension in buffered peptone-water from a 16–24 hr. slant culture. Meningococcus was incubated in an atmosphere of 5 % CO_2 in air. The total N (Kjeldahl) was 2640 μl./ml. while the NH_3 was very low, 14–18 μl./ml. mainly from the peptone which also supplied 495 μl./ml. NH_2-N (Van Slyke).

The "NH_3" medium of Gladstone [1937, 2] for the trained staphylococcus was used with cystine instead of Na thiolacetate as the necessary source of organic S [Fildes & Richardson, 1937]. The organism was maintained on the same medium (medium B) in 5 % CO_2/air. Since growth was slow the inoculum was 0·5–1·0 ml. of 24–48 hr. culture for each 100 ml. medium. Good growth was obtained in 24 hr. by shaking the 100 ml. cultures in 250 ml. conical flasks at 37° in air (110 swings/min., amplitude 3 cm.). Arginine was added when needed as 1·5 ml. 0·5 *M* solution/100 ml. (medium B_A). The trained staphylococcus transferred to medium A or A_G gave good growth in a closed incubator without special pre-

cautions to ensure an adequate supply of CO_2. The trained *Bact. typhosum* was grown on a simpler medium (B′), that of Gladstone [1937, 1] with lactate, no glucose and no $NaHCO_3$.

(3) *Determination of metabolism of washed cells.* The organisms were reaped by centrifuging, washed once with Ringer's solution and resuspended to form a thick suspension. The dry weight was determined by direct weighing on the microbalance of the cells from 0·5 ml. suspension, spun down after dilution with 5 ml. H_2O. The same quantity of suspension was added to neutralized substrate to give 7·5 m*M* (each optical isomer) and phosphate buffer 33·3 m*M* in a final volume of 3·33 ml. The NH_3 was determined initially and at intervals up to 4 hr. on 1 ml. aliquots (0·5 ml. where 2 mol. NH_3 were evolved rapidly) by the method of Parnas & Heller [1924] or that of Conway & Byrne [1933]. The NH_3 was collected in 1 ml. 0·01 *M* H_2SO_4 which was back-titrated with 0·01 *M* NaOH. In the former case the final suspensions were shaken aerobically in tubes ($6 \times \frac{3}{4}$ in.) at 37°. Aliquots were removed at appropriate intervals and washed into the Parnas apparatus with 5 ml. saturated borax. In the latter case, the aliquots were incubated in Conway units already warmed to 37°, with standard acid in the centre well, and 1 ml. saturated K_2CO_3 was added to stop the reaction at the appropriate time and expel the NH_3. It was found advisable to smear lightly the top of the wall of the inner well with semi-solid paraffin fixative to avoid errors due to creeping of solutions. The method was adopted for highly pathogenic organisms like *C. diphtheriae* on account of the simplicity of the apparatus which can be sterilized easily after use by autoclaving. Recoveries of added NH_3 were 97–99 % by either method. Quantities are expressed as μl. gas to facilitate comparison with the usual metabolic quotients (14μg. NH_3-N $\equiv$ $22{\cdot}4\mu$l. NH_3).

Determination of CO_2 output in the breakdown of arginine was by the method of Dickens & Šimer [1931]. Each vessel contained 1·5 ml. Ringer's solution buffered by 25 m*M* $NaHCO_3$ with or without 20 m*M* arginine. Addition of 0·5 ml. organism suspension gave 15 m*M* substrate concentration. The method also showed that there was no O_2 uptake under aerobic conditions.

Identification of the remaining product from arginine is described at the appropriate part of the text.

(4) *Materials.* Synthetic *dl*-amino-acids were used as far as possible to avoid failure to recognize effects due to abnormal optical specificity as shown by the deaminases of animal tissues [Krebs, 1933]. The exceptions were *l*(+)-arginine, *l*(−)-cystine, *l*(−)-histidine, *l*(−)-hydroxyproline, *l*(+)-lysine, *l*(+)-ornithine, *l*(−)-proline and *l*(−)-tryptophan.

Results

The liberation of NH_3 from various substrates by the organisms studied is given in Table 1. The values are average values for a 2–4 hr. period except where there is significant variation with time, when maximum values are given, either initial values (1) or, if an autocatalytic effect is shown, final values (2) for an experimental period not exceeding 4 hr. Further details are considered with reference to the various organisms.

(1) *Streptococci.* Although glucose was known to inhibit almost completely the formation of deaminases in *Bact. coli* [Stephenson & Gale, 1937], this did not seem to be the case with *Str. haemolyticus* Richards since the amount of NH_3 produced in the medium during growth was but slightly affected (Table 2). The slight deficiency of 18μl. equivalent to about 11μg. NH_3-N may well have been used for synthetic purposes since about 150μg. more organisms were formed per

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ml. medium. Similarly with washed suspensions acting on peptone (Table 2) the Q_{NH_3} of glucose-grown organisms, although at first less than with lactate-grown organisms, rapidly rose to a similar level. Owing to the larger crop of organisms the total amount of NH_3-producing enzyme was considerably larger when glucose was present during growth. For this reason, and in order to shorten the lag phase and get more consistent growth, glucose was generally included in the medium for cultivation.

When individual amino-acids were used as substrates, only arginine gave a significant Q_{NH_3} ranging from 48 to 220 μl./mg./hr. with a mean of 119 in 10 experiments. With 21 other amino-acids (the only common ones not tried being hydroxyglutamate and diiodotyrosine) the rate was never more than 2·5% of that with arginine and was, in fact, practically zero within the limits of experimental error. The amino-acids which acted as mutual oxidants and reductants

Table 1. *Liberation of NH_3 from various substrates*

Organism ...	*Streptococcus*						
	1	2	3	4	5	6	7
Medium (see text)	A_G	A_G	A_G	A_G	A_G	A_G	A_G
Glutamine in medium	+	−	−	−	±	±	±
Crop (mg./200 ml.)	28–52	20–47	46	32–43	54	25–104	54–73
Time of growth (hr.)	18	24	24–42	24	42–66	18	18
Q_{NH_3} with:							
0 Substrate	0	—	—	—	0	—	0
I Glycine	2						
Alanine	0						
Valine	1	} 0	1	1	3	1	0
Leucine	1						
Proline	0						
Hydroxyproline	1						
II *iso*Leucine	3						
*nor*Leucine	1	} 2	2	−1	1	−1	1
Phenylalanine	0						
Tyrosine	−2						
III Lysine	0						
Histidine	−1	} 1	0	2	3	0	−1
Ornithine	0						
Tryptophan	1						
Citrulline	2	—	—	—	—	—	—
IV Serine	−1	} 1	2	5	} 1	−2	1
Threonine	0			0			
Methionine	−1	} 0	2	1	2	0	2
Cystine	1						
V Aspartate	0			0		−1	
Glutamate	1	} 1	3	1	} 2	1	} 1
Asparagine	0			1		1	
Glutamine	0	2	2	2	1	2	1
VI Guanidine	−1						
Creatine	1	} −1	1	1	1	1	−2
Urea[1]	−1						
(mol. NH_3)	—	—	—	—	—	—	—
Sulphanilamide	3	2	−4	1	1	0	0
Arginine[1] (range)	49–220	170–184	151–218	103–165	83–110	146–219	200–264
(mol. NH_3, mean)	1·85	1·86	1·82	1·55	1·78	1·96	1·77
NH_3 final in medium (range, μl./ml.)	123–128	132–150	—	114–171	129–139	129–145	129–134
Q_{NH_3} with 1% peptone	62–123	—	—	—	—	—	—

[1] Initial value.

} Vertical braces show mixtures of substrates.

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Table 1 (*cont.*)

Organism ...	*Staphylococcus*							
	8	9	9	9	9	9	9	9
Medium (see text)	A_G	B	B_A	B_A	A_G	A_G	A	A
Glutamine	–	–	–	–	–	–	–	–
Crop (mg./200 ml.)	47–81	24–78	28	50	58	66	14	30
Time of growth (hr.)	18	24–48	24	48	24	72	18	66
Q_{NH_3} with:								
0 Substrate	—	1	4	2	2	2	3	2
I Glycine, Alanine, Valine, Leucine, Proline, Hydroxyproline	3	2	13	30[2]			24[2]	17[2]
II *iso*Leucine, *nor*Leucine, Phenylalanine, Tyrosine	4	1	7	5			5	2
III Lysine, Histidine, Ornithine, Tryptophan	4	1	4	6	28[2]*	22*	5	4
Citrulline	—	—	—	—			—	—
IV Serine	13							
Threonine	7	4	8	20			12	19
Methionine, Cystine	6							
V Aspartate, Glutamate	7	2	5	6			5	3
Asparagine		—	—	—			—	—
Glutamine	5	—	—	—			—	—
VI Guanidine	2	1	2	3			3	2
Creatine	3							
Urea[1]	42	>200	>350	*ca.* 1300	*ca.* 900	5	>47	29[2]	41
(mol. NH_3)	—	1·96	1·96	2·05	—	2·26	—	—
Sulphanilamide	4	—	—	—	—	—	—	—
Arginine[1] (range)	85–200	3–6	18	20	28	8	217	35
(mol. NH_3, mean)	1·95	—	—	—	1·93	—	1·92	—
NH_3 final in medium (range, μl./ml.)	131–144	—	—	—	19	248	148	249
Q_{NH_3} with 1% peptone	—	—	—	—	—	—	—	—

[1] Initial value, except where otherwise indicated.
[2] Maximum value reached autocatalytically.
} Vertical braces show mixtures of substrates.
* Citrulline, asparagine and glutamine omitted.

in the reaction of anaerobes discovered by Stickland [1934], also gave no enhanced NH_3 formation when combined in all possible pairs (Table 3). The naturally occurring amides, asparagine and glutamine, yielded no NH_3, nor did guanidine, creatine or urea which were tested on account of their chemical relationship to arginine. The suspensions used in all these cases were highly active when tested with arginine. Sulphanilamide gave no NH_3 and did not affect the breakdown of arginine (Fig. 1).

Other streptococci with less fastidious growth requirements were tested to find if limited power to form NH_3 was associated with the need for glutamine during growth. To reduce labour on account of the possibility of large numbers of negative results, mixtures of amino-acids were used in the first place, individual

Table 1 (*cont.*)

Organism ...	*Meningo-coccus*	*Bact. typhosum*			*C. diph-theriae*
	10	11	11	12	13
Medium (see text)	A_S	A	A_G	B′	A_G
Glutamine in medium	–	–	–	–	–
Crop (mg./200 ml.)	6–18	44–60	70	22–44	22–64
Time of growth (hr.)	24	24	24	24	24–48
Q_{NH_3} with:					
0 Substrate	0–11	1	—	1	−2
I Glycine, Alanine, Valine, Leucine, Proline, Hydroxyproline	8–13	30	—	4	−1 to +4
II *iso*Leucine, *nor*Leucine, Phenylalanine, Tyrosine	0–7	4	—	0	−2 to +7
III Lysine, Histidine, Ornithine, Tryptophan	2–4†	12–29†	—	2	0–2†
Citrulline	—	—	—	—	—
IV Serine	9–11	204–280[1]	0	30–64[2]	3
Threonine		11–57[2]	—	4–10	4
Methionine		−1	—	3	0
Cystine		−1	—		−1
V Aspartate	7	183[1]	55[1]	14–25	7–48[1]
Glutamate		8	—	3	1
Asparagine	—	—	—	—	—
Glutamine	—	—	—	—	—
VI Guanidine, Creatinine, Urea[1]	—	—	—	—	—
(mol. NH_3)	—	—	—	—	—
Sulphanilamide	—	—	—	—	—
Arginine[1] (range)	0–2	10–24	—	−1 to 1	−3 to 11
(mol. NH_3, mean)	—	—	—	—	—
NH_3 final in medium (range, μl./ml.)	28–67	208–216	36–43	—	38–62
Q_{NH_3} with 1% peptone	14–24	—	—	—	32

[1] Initial value.
[2] Maximum value reached autocatalytically.
} Vertical braces show mixtures of substrates.
† Arginine included.

Table 2. *Effects of lactate and glucose in growth medium on NH_3 production by* Str. haemolyticus *Richards*

Medium, with glutamine	A	A_L	A_G
Final NH_3, μl./ml.	146	141	128
Crop, mg./100 ml.	5	7	20
Q_{NH_3}, washed cells in 5% peptone			
0–30 min.	—	480	290
30–90 min.	—	500	455

Table 3. *Absence of Stickland reaction from* Str. haemolyticus *Richards*

Q_{NH_3} in the presence of hydrogen acceptors and donators combined in pairs

		Donators		
Acceptors	—	Alanine	Valine	Leucine
—	0	0	0·5	1
Glycine	2	0	0·5	−0·5
Proline	0	0	0	0
Hydroxyproline	1	0	1	0·5

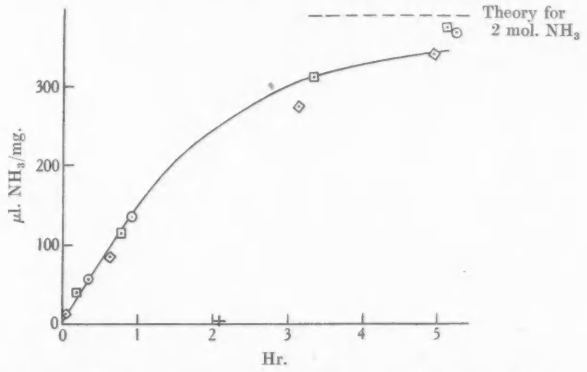

Fig. 1. NH_3 production with arginine and sulphanilamide by *Str. haemolyticus* Richards strain. ⊙ Arginine 7·5 m*M*. ⊡ Arginine 7·5 m*M* + sulphanilamide 7·5 m*M*. ◇ Arginine 7·5 m*M* + sulphanilamide 1·5 m*M*. + Sulphanilamide 7·5 or 1·5 m*M*.

compounds of a group being examined subsequently only if significant amounts of NH_3 were produced. Most mixtures contained only chemically related compounds but the acids which take part in the Stickland reaction were grouped together on account of possible interactions. To reduce the total number of mixtures phenylalanine and tyrosine were included in group II and trytophan in group III. Apart from slight action on serine by one of the haemolytic streptococci of group B similar results were obtained. Growth of the strain of *Str. viridans* was slow but was not improved by glutamine though Fildes & Gladstone [1939] found that the compound was needed by younger strains supplied by Dr Colebrook. The faecal streptococcus freshly isolated from human urine grew better with glutamine at first, but after a few subcultures on nutrient agar the organism dispensed with the need for the addition of this factor to medium A_G. The breakdown of arginine was unaffected by these nutritional changes and no other amino-acids yielded NH_3 in significant amount. Although this organism appeared to have developed the ability to synthesize glutamine during growth none was produced from NH_4^+ glutamate in washed suspension since no NH_3 disappeared.

From the representative strains examined it seemed probable that this breakdown of arginine was a constant character of streptococci. The activity per unit weight of organism was not widely influenced by the presence of arginine or glucose in the growth medium (Table 4), but the mass of growth was increased especially by glucose, so that the enzyme responsible appeared to be constitutive, the total amount formed depending on the amount of organism produced rather

Table 4. *Effect of arginine in growth medium on NH_3 production by* Str. haemolyticus *Richards*

Medium, with glutamine	A	A_A	A_G	A_{AG}
Crop at 20 hr. mg./100 ml.	5	7	12	16
Q_{NH_3}, washed cells, 7·5 m*M* arginine	141	138	155	169
NH_3 in growth medium, μl./ml.				
20 hr.	147	271	118	331
44 hr.	146	323	123	340
Extra due to arginine	177		217	
Mol.	1·58		1·84	

than directly on the materials in the medium available for its synthesis. The yield of NH_3 from arginine in the medium approached 2 mol. in the presence of glucose and though the amount was less in its absence the reaction may not have been complete. All the streptococci produced nearly 2 mol. NH_3 from arginine in washed suspension in cases where the reaction was followed to completion. The lowest yield was 1·55 mols. with one of the haemolytic streptococci of group B (No. 4). Further reaction after adding more substrate showed that stoppage was not due to destruction of the enzyme or development of an adversely high *p*H.

Manometric experiments with *Str. faecalis* and *Str. haemolyticus* Richards showed that 1 mol. CO_2 was produced with 2 mol. NH_3. A typical experiment is shown in Table 5. Aerobically the Q_{O_2} was negligible and the same reaction

Table 5. *CO_2 and NH_3 production from arginine by* Str. haemolyticus *Richards*

Substrate, 200 μl. per mg. dry cells. Time for completion, 120 min. after 20 min. equilibration. NH_3 from substrate during equilibration, 110 μl. per mg.

		Metabolism, μl./mg.				
Gas phase	Substrate	O_2	CO_2	NH_3	$\frac{NH_3}{\text{Arginine}}$	$\frac{CO_2}{NH_3}$
5% CO_2 in 95% O_2	—	−10	+ 10	0	1·81	0·41
	Arginine	− 5	+115	+252		
5% CO_2 in 95% O_2	—	—	+ 8	0	1·79	0·48
	Arginine	—	+126	+248		

occurred anaerobically. A disadvantage of the method used, that of Dickens & Šimer [1931], was that substrate could not be added to the medium after equilibration with gas mixture in the thermostat. The NH_3/arginine ratio therefore referred to the whole of the reaction from the time of immersion of the vessels in the bath, while the CO_2/NH_3 referred to the last 70% of the reaction (in the example given) occurring after equilibration. The figures do, however, suggest that the reaction is:

$$H_2NC(:NH).NH(CH_2)_3CHNH_2COOH + 2H_2O = H_2N(CH_2)_3CHNH_2COOH + 2NH_3 + CO_2. \quad (1)$$

To confirm this equation NH_2-N determinations were made and the formation of ornithine was established by benzoylation followed by isolation of ornithuric acid. *Str. haemolyticus* Richards, from 4 flasks of medium A_G with glutamine, was suspended in $NaHCO_3$ and added to arginine in 0·9% NaCl to give 30 m*M* $NaHCO_3$ and 15 m*M* arginine (Ringer's solution was avoided here and in washing the organisms because of the complication of precipitation of Ca and Mg when the liquid was made alkaline in the subsequent benzoylation). The final suspension was shaken at 37° in 2 Krebs' vessels in equilibrium with 5% CO_2 till the

reaction was complete as shown by the CO_2 absorption (corrected for no substrate control) in a sample shaken under the same conditions in a Warburg manometer vessel (2–3 hr.). The organisms were killed by acidification with 0·1 vol. *N* acetic acid and heating for 5 min. on the water bath. NH_3 was removed from 4 ml. clear filtrate by evaporation to small bulk with a few drops excess $Mg(OH)_2$ suspension and the sample was acidified with acetic acid and transferred quantitatively to the Van Slyke apparatus for NH_2-*N* determination. The results, given in Table 6, show clearly that the NH_2-*N* is doubled in spite of the loss of 2 mol.

Table 6. *Amino-N and NH_3 production from arginine by* Str. haemolyticus *Richards*

	Initial		Final	
	μl./ml.	mol.	μl./ml.	mol.
Arginine	338	1·00	0	0
NH_2-N	365	1·08	715	2·12
NH_3	22	0·06	630	1·87

NH_3, i.e. ornithine must be formed. Complete decomposition of arginine was confirmed by addition of 0·5 vol. 5% flavianic acid to another small sample; no flavianate was precipitated. The remainder of the acid filtrate (45 ml.) was therefore evaporated to dryness, dissolved in a small volume of 2*N* NaOH and freed from NH_3 in a vacuum desiccator. The product of benzoylation (Schotten-Baumann) was transferred quantitatively to a Soxhlet thimble (10 × 50 mm.), which was used as a filter. After drying, benzoic acid was extracted with ether in an all-glass continuous extractor. Subsequent extraction with alcohol gave 211 mg. material, M.P. 179–181°, yield 98%. A single crystallization from 8 ml. 50% alcohol gave 114 mg. fairly pure ornithuric acid, M.P. 180°, yield 67%; mixed M.P. 182° with a specimen, M.P. 185°, prepared in similar yield from *l*(+)-ornithine (Hofmann-La Roche). Ornithuric acid was prepared similarly from the products of the action of the faecal streptococcus on arginine and crystallized to constant M.P. 185–186°, mixed M.P. 184°. All M.P. are uncorrected. The M.P. of ornithuric acid is given variously in the literature, 182–189°.

(2) *Staphylococci*. The main difference between staphylococcus (No. 8) and streptococcus, both growing on the peptone medium A_G, was the urease activity of the former but the rate was less than the rate of attack on arginine. The staphylococcus had also small but definite actions on serine, threonine, glutamine and possibly other substrates. With the trained organism grown on the "NH_3" medium urease activity became predominant, accurate measurement not being made under the conditions chosen owing to disappearance of much of the substrate before taking the second sample. In order to find if the almost complete disappearance of the arginine enzyme was due to the absence of the substrate during growth the organism was grown in the medium B_A with the addition of arginine, but little of the enzyme was produced even if the period of growth was prolonged to 48 hr. and urease activity was still very high. The activity with some amino-acid mixtures was of the same order as that with arginine. If the organism was transferred to the peptone medium for streptococcus, arginine was attacked in washed suspensions after 24 hr. growth with glucose in the medium (A_G), but the enzyme was probably not maximal since practically no NH_3 had been formed during growth. The urease had reverted to a position of less importance quantitatively. If the incubation in the growth medium was prolonged to 72 hr. nearly twice as much NH_3 was liberated as by any other coccus in this

medium, the arginine enzyme had decayed considerably and the urease showed increased activity. In both cases the NH_3 from a complete mixture of all the other compounds used was relatively high (the smaller groups were not tried). In order to enhance this activity, if possible, the organism was grown on the peptone medium without glucose. There was little change, but the usual amount of NH_3 had been liberated during 18 hr. growth and the arginine enzyme was by then highly active. More NH_3 had been liberated in the growth medium after 66 hr. and the arginine enzyme had decayed to 15% of its former value, with some increase in the urease.

(3) *Other organisms.* The Coccaceae so far shown to attack arginine were all gram-positive. For comparison the gram-negative meningococcus was studied. The values were less accurate than the others owing to poor growth of the organism, only 0·15–0·30 mg. dry cells per ml. final suspension being available instead of 1–2 mg. The extreme ranges of values are given rather than the means. No amino-acid mixture gave much NH_3, but as a small amount of NH_3 was liberated in the medium during growth, peptone was used as a standard substrate to show that each suspension used was active. Washed cells had the same activity as unwashed cells and the activity was not enhanced by the addition of serum, which was needed for growth. The feeble activity with amino-acids was therefore not due to damage to the enzyme systems of the delicate organism by Ringer's solution.

Bact. typhosum was studied as a pathogenic member of the Bacteriaceae. The stock strain gave much NH_3 in the medium in the absence of glucose but little in its presence. The organisms were therefore usually grown without glucose. With washed suspensions substrates showing highest activity were serine and aspartate. Arginine, threonine and Stickland's acids (group I) had significant activity, but methionine and cystine were inactive. Glucose in the growth medium inhibited the formation of the serine enzyme completely and that of the aspartate enzyme by 70%. The organism therefore resembled in deaminating power the related *Bact. coli* [Stephenson & Gale, 1937; Gale & Stephenson, 1938; Gale, 1938]. The trained strain showed more feeble activity with little relative change in the rates of attack of the various substrates, excepting possibly the slightly enhanced activity with cystine or methionine.

Arginine thus appeared to be of less importance in gram-negative organisms, quantitatively at any rate, than in the gram-positive cocci. As a gram-positive pathogen of a different order, *C. diphtheriae* was chosen for study. The little NH_3 formed during growth was not affected by the presence of glucose or lactate in the medium. Growth was best with glucose and glucose-grown organisms were used for the experiments with washed suspensions. Little activity was shown except with aspartic acid.

Discussion

The decomposition of arginine studied here appears to be due to a distinct enzyme and not due to the action of urease following that of arginase (using the term in the accepted sense first used by Kossel & Dakin [1904]). The reasons for this are:

(1) The strains of streptococci used have been shown to possess no urease.

(2) The urease of the stock strain of staphylococcus is insufficiently active to account for the rate of breakdown of arginine.

(3) In a strain of staphylococcus trained to grow on an "NH_3" medium the urease activity can be varied at will between very wide limits, the arginine enzyme varying in an inverse manner. The reciprocal relationship suggests that the enzymes may be related though not identical.

The enzyme could be described as an "arginase", but in order to specify the reaction process the name "arginine dihydrolase" is suggested. This distinguishes the enzyme from "arginase", in the accepted sense, which could be described on the same basis as "arginine monohydrolase".

Descriptions of bacterial arginase in the literature may refer often to arginine dihydrolase when the analytical methods used have shown only the increase in NH_2-N (Hino [1924] on *Ps. pyocyanea* and *Ps. fluorescens*) or disappearance of arginine (Kossel & Curtius [1925] on *Ps. pyocyanea*). Both organisms show urease activity [Hino, 1924]; both are gram-negative rods and may be feebly pathogenic. Other organisms which may possess arginine dihydrolase are *Cl. sporogenes* and *Cl. histolyticum*, gram-positive rods which attack arginine more actively than other amino-acids, but differ from the gram-positive cocci in deaminating very actively alanine and glutamate with less action on tyrosine and cystine [Bessey & King, 1934]. *Cl. sporogenes* has also been shown to differ in the mutual oxidation and reduction in pairs of amino-acids [Stickland, 1934]. Arginine moreover yields 3 mol. NH_3 either alone or as a H-donator and ornithine is attacked either alone or as a H-acceptor giving NH_3 and δ-aminovaleric acid [Woods, 1936]. Since urea is not attacked either alone or with H-acceptors or donators, it seems likely that arginine can break down as in the gram-positive cocci to ornithine, which is then attacked further only by the anaerobes.

The mechanism of the action of arginine dihydrolase has not been studied in detail, but citrulline cannot be an intermediate with *Str. haemolyticus* Richards and free urea has also been shown not to be affected. Since the known mechanisms of enzyme reactions generally involve simple stages like the addition or removal of H_2, H_2O or NH_3, it can only be suggested that there is formed a tautomeric form of urea, $HN{:}C(-O^-).\overset{+}{N}H_3$, or its ion, $HN{:}C(-OH).\overset{+}{N}H_3$, such as was postulated by Werner [1923], and instead of being liberated from the enzyme surface in a free state it is further activated to give NH_3 and CO_2 by further reaction with H_2O or OH^-.

Since it has been generally supposed that the deaminases may be involved *in vivo* in synthesis of amino-acids on the basis of the reversibility of enzyme reactions, it was at first surprising that the deaminating power was low in those strains of *Staph. aureus* and *Bact. typhosum* which had been trained to synthesize their amino-acid requirements from NH_3. In the animal, however, the amino-acid oxidases react with O_2 through flavin-adenine dinucleotide [Warburg & Christian, 1938] and as nothing is known of the presence of the nucleotide in the bacteria examined, it may be a limiting factor in deamination. The enzyme may also be linked through coenzyme I with other dehydrogenase systems, such as in the lactic or malic system [Dewan, 1939]. The latter, in conjunction with fumarase, is certainly of importance in the oxidation of *l*(+)-glutamate by *Bact. coli* [Krebs, 1937]. The possibility must also be considered of interaction with α-keto-acids without loss of NH_3, i.e. the transamination reaction of Braunstein & Kritzmann [1937, quoted by Cohen, 1939]. The failure of oxidative deamination in the organisms tested or even the failure of amino-acids to enable streptococci to reduce indigo-tetrasulphonate, may therefore be due to the absence of other factors than the enzyme proper.

The function of arginine dihydrolase in bacteria must remain a matter for future study, but five possibilities may be suggested.

(1) *A neutralization mechanism.* The earlier production of the enzyme by the trained staphylococcus growing on peptone without glucose makes this unlikely, contrasting with the conditions for the formation of the decarboxylating enzymes in *Bact. coli* [Gale, 1940].

(2) *A mechanism for supplying NH_3 for synthesis.* The synthesis of amino-acids, especially those which Gladstone [1937, 2] found more easily dispensable, may be of importance in the natural habitat of the organism where keto-acids with the appropriate C-skeletons may be available for reductive amination, possibly linked with the energy-yielding reactions of carbohydrate metabolism through coenzyme I.

(3) *Ornithine production.* This is suggested by the effects of lack of arginine and addition of ornithine in the growth of *Str. haemolyticus* in a synthetic medium [McIlwain, 1940]. The suggestion of the possibility of the oxidation of ornithine to glutamine by keto-acids in liver [Bach, 1939] affords an interesting speculation as to the relation between the two in streptococci.

(4) *Toxic effect of NH_3 on the host.* Tauber & Kleiner [1931] showed that crystalline urease produces symptoms resembling NH_3 poisoning. The minimum lethal dose per g. in mice gave *in vitro* 1·1 mg./hr. NH_3-N at 20°. Table 1 shows that *Str. haemolyticus* Richards would produce about 0·03–0·14 mg./hr./mg. dry cells at 37°. In comparing these figures we should consider (*a*) multiplication of the cells in the tissues of the host, (*b*) activation of the organism by coenzymes in the body fluids of the host, (*c*) the frequent localization of streptococcal and staphylococcal lesions.

(5) *Relationship to staphylococcal α-haemolysin.* Neither haemolysin [Gladstone, 1938] nor enzyme is produced on the "NH_3" medium. Although arginine is quantitatively the most important amino-acid for haemolysin production (optimum 6·6–20 m*M*) others are also required; nor is the addition of arginine alone to the medium (7·5 m*M*) a sufficient condition for the production of the enzyme. Fildes [1934], discussing bacterial enzyme variation, has distinguished between (*a*) catabolic enzymes rapidly formed through the stimulus of the appropriate substrates and (*b*) anabolic enzymes for the synthesis of an essential nutrient produced through lack of the nutrient only after a lengthy training. The arginine enzyme may therefore be specially associated with pathogenicity since it cannot be classified as an adaptive catabolic enzyme of type (*a*). On the other hand the great activity normally found in untrained organisms does not appear to be essential for the life of a gram-positive coccus, yet full activity is restored in a strain which had lost it by a single subculture on an appropriate medium.

Summary

1. Gram-positive cocci contain an enzyme which attacks arginine in accordance with equation (1) with a Q_{NH_3} *ca.* 50–200.

2. The enzyme has been named arginine dihydrolase to distinguish it from arginase with which it is not identical.

3. With a trained strain of staphylococcus the arginine dihydrolase content can be varied according to the conditions of growth between Q_{NH_3} 217 and 3 while the urease content varies reciprocally between Q_{NH_3} 5 and 1300.

4. Possible functions of arginine dihydrolase in the life of the organism are discussed, especially in relation to pathogenicity.

5. Gram-positive cocci produce no NH_3 aerobically from other amino-acids except, with staphylococcus, serine, Q_{NH_3} 13, and threonine, Q_{NH_3} 7.

6. Meningococcus has, aerobically, Q_{NH_3} 14–24 on 1% peptone but gives no significant deamination with amino-acids.

7. *Bact. typhosum* deaminates aerobically serine, aspartate and, to some extent, threonine and arginine with Q_{NH_3} 240, 180, 30 and 15 respectively. The

formation of serine deaminase is inhibited 100% and that of aspartase 70% by 12·5 m*M* glucose in the growth medium.

8. *C. diphtheriae* has an aerobic Q_{NH_3} 30 with aspartate but gives no significant deamination with other amino-acids.

I am indebted to Dr P. Fildes under whose inspiration and guidance this work progressed and to my colleagues for helpful discussion, especially to Dr G. P. Gladstone who gave active help with the bacteriological work in the early stages. I wish to thank Mr D. E. Hughes for Van Slyke determinations.

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129. THE METABOLISM OF ARTICULAR CARTILAGE

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(*Received 29 May 1940*)

THIS work is a continuation of that of Bywaters [1937] who showed that cartilage has practically no respiratory or oxidative enzymes but has definite glycolytic and dehydrogenase activities. The object of the present work was to determine the function of the dehydrogenase in the absence of oxidase. After getting indications that it played a part in the glycolytic mechanism, further study of glycolysis was made. While the work was being prepared for publication, a paper by Lutwak-Mann [1940] has appeared dealing with different aspects of cartilage glycolysis, especially the role of possible phosphorylated intermediates and coenzymes.

Methods

Manometric estimations were carried out as described by Dixon [1934] except that for the small O_2 uptakes measured "KOH-papers" were unnecessary and were avoided in order to decrease the blanks. To obtain sufficient material, the carpo-metacarpal joints of freshly slaughtered horses were used for most experiments and except where otherwise stated all data refer to this material. The medium was Ringer solution buffered to *p*H 7·3 by NaH_2PO_4-Na_2HPO_4 for O_2 uptake or $NaHCO_3$-CO_2 for manometric estimation of acid production. Except where other details are given the buffer concentration was 0·2 vol. isotonic solution and glucose was added to give a final concentration of 0·2%. Further details are as given by Bywaters [1937]. Manometric methods were supplemented by chemical estimations of bisulphite-binding substances (BBS) by the method of Clift & Cook [1932] and lactic acid by that of Friedemann & Graeser [1933].

Metabolic quantities are expressed, for convenience, as μl./g. dry tissue, i.e. the Q values given here should be divided by 10^3 for comparison with the usual metabolic quotients for more active tissues.

Results

(1) *Respiration.* Table 1, summarizing the results of a large number of experiments, shows that the respiration of horse articular cartilage is definite though small. The standard deviation indicates there is a 1 in 3 chance of the Q_{O_2} lying between -3 and -17 μl./g./hr. and a 21 in 22 chance between $+4$ and -24. Actually the 80 recorded values ranged from 0 to -39. These values should perhaps be reduced slightly since controls with the same amount of medium (5 ml.) and glass wool in place of tissue to prevent excessive splashing of this unusually large amount showed a small fall in pressure equivalent to *ca.* -4 μl./g./hr. This is presumably due to absorption of the last traces of CO_2 in the vessel. Table 1 also extends the observations of Bywaters [1937] on the increase of O_2 uptake by methylene blue. The optimum effect occurs in the neighbourhood of 10^{-4} M but varies with the sample of tissue. Slight inhibition

Table 1. *O_2 uptake of articular cartilage and the effect of methylene blue*

	Methylene blue concentration ($\times 10^{-5}$ *M*)					
	0	1	3	7	10	100
(*a*) Horse						
Mean Q_{O_2} (μl./g./hr.)	10	42	65	72	88	64
No. of observations	80	7	28	6	23	3
Standard deviation	7	20	37	13	20	7
Mean control (no tissue)	3	—	4	—	5	4
No. of observations	9	—	4	—	5	2
Standard deviation	4	—	5	—	7	4
(*b*) Rabbit						
Mean Q_{O_2} (μl./g./hr.)	150	—	450	—	350	—
No. of observations	4	—	4	—	4	—
Standard deviation	35	—	60	—	60	—

Table 2. *Effect of 2:4-dinitro-*o*-cresol on O_2 uptake*

		Q_{O_2} (μl./g./hr.)				
		Conc. of 2:4-dinitro-*o*-cresol ($\times 10^{-5}$ *M*)				
Exp.	State of cartilage	0	1	3	10	100
15/3	Normal	5	16	18	26	9
22/3	Normal	13	—	—	25	17
1/4	Eroded	5	—	—	6	2
5/4	Normal	8	—	—	21	6

occurs with 10^{-3} *M* but this concentration is not highly toxic as in kidney [Dickens, 1936]. Dinitro-*o*-cresol also catalyses the O_2 uptake at similar concentrations with normal tissue (Table 2), but the effect is very much smaller. This supports the view that cartilage must respire to a small extent since nitrophenols exert their effect only on tissues with a complete respiratory system [Greville & Stern, 1935]. The rate with high concentrations of methylene blue was not limited by diffusion into the tissue since increase of the usual average thickness of the slices from 0·24 to 0·65 mm. reduced the O_2 uptake only insignificantly from -112 to -102.

Articular cartilage from rabbits had a much greater O_2 uptake than tissue from the horse (Table 1); fibrous cartilage (rabbit meniscus) similarly gave a value -30, increased to -110 and -140 by 3 and 10×10^{-5} *M* methylene blue respectively. This material was, however, less suitable for systematic investigation on account of the number of animals, labour and time involved in preparing sufficient for use in the Warburg apparatus. The micro-respirometer of Heatley *et al.* [1939] would be more suitable for it. The values approach those of Dickens & Weil-Malherbe [1936] for rat costal cartilage, 220–680.

(2) *Dehydrogenase activity.* Since respiration is normally small, the optimal O_2 uptake in the presence of methylene blue is a measure of the activity of complete dehydrogenase systems (i.e. enzyme-coenzyme-diaphorase) in the tissue. To get some idea of the substrates used, the effects of glucose, lactate and pyruvate were tested (Table 3) after a period of substrate deprivation, which supplied a control figure for the same sample of tissue when corrected for the slight spontaneous fall with a similar sample of untreated tissue. Glucose and lactate had no effect and pyruvate was slightly inhibitory in the experiment quoted. In other experiments it appeared that lactate was most efficient in maintaining O_2 uptake at its initial level, while the fall in the presence of pyruvate might be as much as 60%. It is evident that the tissue itself provides ample substrate for maximum dehydrogenase activity. Lactic dehydrogenase seems to be involved

Table 3. *Effects of substrates and other reagents on O_2 uptake with or without methylene blue*

Exp.	Reagent	Conc. 10^{-3} *M*	Dye conc. 10^{-5} *M*	Q_{O_2} (μl./g./hr.) Control	With reagent	% change
*8/6	Pyruvate	25	0	36	40	+11
	,,	25	3	109	94	−14
	l(+)-Lactate	25	3	109	111	+ 2
	Glucose	12·5	3	120	119	− 1
†12/8	Ca^{++}	2	3	83	84	+ 1
	,,	2	10	112	117	+ 5
†18/1	F^-	2	3	36	38	+ 6
	,,	6	3	33	44	+33
	,,	12	3	37	50	+35
†22/1	,,	2	10	68	69	+ 1
	,,	6	10	45	46	+ 2
	,,	12	10	51	43	−16
27/1	HCN	1	7	50	64	+28
19/2	,,	10	10	68	91	+34
22/2	*n*-Heptyl alcohol	*ca.* 12 (sat.)	10	108	17	−84
‡24/2	,,	*ca.* 12 (sat.)	10	86	6	−93
30/4	*o*-Iodoxybenzoate	1	0	31	29	− 7
	,,	1	3	125	121	− 3
4/5	,,	10	0	7	8	+14
	,,	10	3	85	55	−35

* No glucose present. † Using Ca^{++}-free Ringer.
‡ Cartilage showing erosion and fibrillation.

on account of the pyruvate inhibition [Green & Brosteaux, 1936]. Bywaters [1937] showed that the rate of lactate production either with or without added glucose was adequate to account for the O_2 uptake now observed.

The effect of various reagents on the process is also shown in Table 3. No constant inhibition was produced by fluoride, and with a methylene blue concentration barely sufficient to induce maximal O_2 uptake a small but definite rise was observed. The table also shows that the necessary omission of Ca^{++} from the Ringer in these experiments does not affect the values. The effect of HCN is of greatest interest since the value was increased by about a third, suggesting the removal of a ketonic oxidation product as cyanohydrin [Wendel, 1934; Green & Brosteaux, 1936]. The precautions of Krebs [1935] to prevent loss of HCN to the absorbing KOH were adopted. As with *n*-heptyl alcohol which was a powerful inhibitor, it was only possible to determine controls on separate samples of tissue in different vessels, owing to the volatility of the reagents. The results are therefore less accurate than with other reagents on account of the difficulty of getting identical samples with such large quantities of tissue. The effect of *o*-iodoxybenzoate was tested since its use in the treatment of arthritis suggested the possibility of a specific effect on cartilage metabolism. It caused some inhibition in the 1st hr. in the experiment quoted but was effective only at high concentration. Other experiments showed that the inhibition increased to about 50 % in the 2nd hr. with 10 m*M*.

Further evidence for the production of pyruvate was the positive nitroprusside reaction [Simon & Piaux, 1924] in the vessel contents, after the removal of methylene blue with kaolin, only at the end of a long experiment (5 hr.). The

BBS formation, determined also after the removal of methylene blue, was inadequate to account for the O_2 uptake (Table 4, exp. 19/12) according to equation (1) which is discussed later. The production of CO_2 aerobically in the presence of methylene blue was always observed, though the accuracy was much less than is usual in the determination by the phosphate method of Dickens & Šimer [1930] owing to the large blank, determinable with an error as large as

Table 4. *Pyruvate metabolism and lactate formation*

Exp.	Conditions		Metabolite	Initial µl.	Final µl.	µl./g./hr.
19/12	Glucose, 5 hr.	O_2	CO_2	42	88	+ 39
	Tissue, 230 mg. dry wt.	MeB, 10^{-4} *M*	O_2	—	—	− 54
			BBS	32	75	+ 36
11/1	Pyruvate, 4 hr.	N_2	CO_2	64	80	+ 14
	Tissue, 275 mg. dry wt.		BBS	109	99	− 9
			Volatile BBS	2	2	0
4/1	Glucose, 4 hr.	N_2	CO_2	57	79	+ 20
	Tissue, 270 mg. dry wt.	MeB, 10^{-4} *M*	Lactate	60	204	+133
		O_2	CO_2	77	65	− 11
			O_2	—	—	− 22
			Lactate	42	234	+178
		O_2	CO_2	57	98	+ 38
		MeB, 10^{-4} *M*	O_2	—	—	− 89
			Lactate	120	388	+248

± 15 %. This was unavoidable owing to the difficulty of washing CO_2 completely from the large samples of tissue, although that in the medium was kept as low as possible by reducing the phosphate concentration to *M*/60. The CO_2 formed cannot arise by simple decarboxylation of pyruvate, since there was no anaerobic formation of volatile BBS (Table 4, exp. 11/1; solution made acid to Congo red, added acetaldehyde giving 98 % recovery). In this experiment the total BBS disappearance was small and CO_2 formation insignificant. It therefore seems most likely that CO_2 formed aerobically arises by oxidative decarboxylation of pyruvate, perhaps catalysed by methylene blue. Such oxidative decarboxylations are of widespread occurrence in animal tissues and bacteria with O_2 [Long, 1938; Barron & Lyman, 1939], dyes [Lipmann, 1937] or even a molecule of the same or another ketonic acid [Krebs & Johnson, 1937; Krebs, 1937; Lipmann, 1939] as oxidant. If O_2 is the ultimate oxidant the oxidation of lactate may occur in accordance with the scheme (quantitative data from Table 4, exp. 19/12):

$$\underset{}{CH_3.CHOH.COOH} + \tfrac{1}{2}O_2 = \underset{}{CH_3.CO.COOH} + H_2O \qquad (1)$$
$$\underset{}{\quad} \quad -34{\cdot}5 \quad\quad +69$$
$$\underset{}{\quad} \quad \text{(calc.)} \quad\quad \text{(calc.)}$$

$$\underset{}{CH_3.CO.COOH} + \tfrac{1}{2}O_2 = \underset{}{CH_3.COOH} + CO_2 \qquad (2)$$
$$\underset{}{-39} \quad\quad -19{\cdot}5 \quad\quad\quad +39$$
$$\underset{}{\text{(calc.)}} \quad\quad \text{(calc.)} \quad\quad\quad \text{(obs.)}$$

Net formation of pyruvate = 30 µl./g./hr. (calc.)
Formation of BBS = 36 µl./g./hr. (obs.)

The O_2 uptake and loss of pyruvate in reaction (2) were calculated from the CO_2 formed and the remainder of the observed O_2 uptake was assumed to be due to the formation of pyruvate from lactate according to equation (1). The net calculated pyruvate formation agreed very well with the BBS found experi-

mentally. Direct confirmation of this hypothesis by determination of volatile acid was not possible on account of the difficulties in determining the small amount expected (e.g. about 4 ml. 0·5 mM per vessel in the exp. quoted). It seems unlikely that much of the BBS was methylglyoxal although this was formed from hexosediphosphate by calf cartilage [Lutwak-Mann, 1940], since it would require even more complete oxidation of some other substrate to account for the O_2-uptake, presumably with greater evolution of CO_2.

(3) *Anaerobic glycolysis.* It had been observed that lactate production (determined chemically) was greater aerobically than anaerobically when the O_2-uptake was above the average, and that it was further increased by methylene blue (Table 4, exp. 4/1). A decrease had been expected according to the hypothesis that lactate was oxidized to pyruvate under these conditions. Bywaters [1937] had found only irregular differences between aerobic and anaerobic glycolysis but the lactic acid formed was equivalent to the CO_2 liberated from $NaHCO_3$ buffer. The effect of pyruvate on glycolysis was therefore studied manometrically (Fig. 1). There was little effect in the absence of glucose, but in

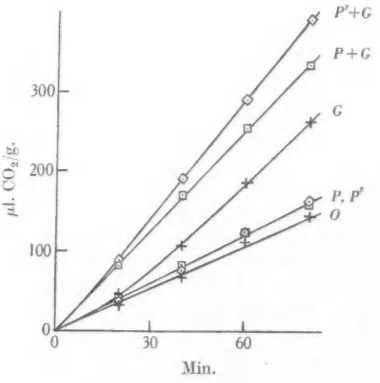

Fig. 1.

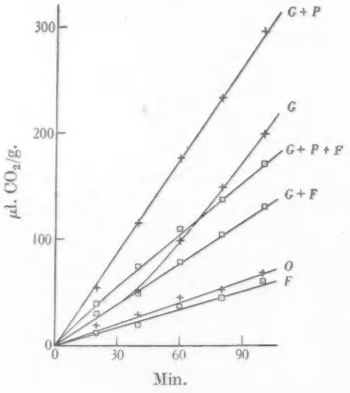

Fig. 2.

Fig. 1. *The effect of pyruvate on glycolysis.* Compounds added at 0 min. to give final concentration: G=glucose, 10 mM; P=pyruvate, 2 mM; P'=pyruvate, 0·2 mM. 60 min. preliminary anaerobiosis.

Fig. 2. *The effect of fluoride on glycolysis and its pyruvate catalysis.* Compounds added at 0 min. (after 60 min. preliminary anaerobiosis) to give final concentration: G=glucose, 12·5 mM; P=pyruvate, 1 mM; F=fluoride, 7 mM.

the presence of that substrate, after a period of anaerobiosis without it, the induction period of 30–50 min. normally needed to reach the maximum rate was completely abolished by pyruvate at as low a concentration as 0·2 mM. The effect was catalytic since (*a*) 0·2 mM pyruvate, *ca.* 75 μl./g. tissue, produced more than 130 μl./g. extra CO_2, (*b*) higher pyruvate concentration was slightly inhibitory, (*c*) pyruvate without glucose produced little extra CO_2. Pyruvate is apparently a normal intermediate in cartilage glycolysis, but is not normally present in sufficient amount for maximal glycolysis in the presence of added substrate. Since Bywaters [1937] found that the glycolysis is less sensitive to fluoride in the absence than in the presence of added glucose, the effects of fluoride were studied

further (Fig. 2). Pyruvate catalysis was largely abolished and the results of Bywaters were confirmed.

The effects of other inhibitors were also studied (Table 5). Iodoacetate caused 50 % inhibition at 0·017 mM and almost complete inhibition at mM. Inhibition was also almost complete with *n*-heptyl alcohol and *o*-iodoxybenzoate, but at

Table 5. *Effect of inhibitors on glycolysis*

Exp.	Inhibitor	Conc. 10^{-3} M	$Q_{G}^{N_2}$ (µl./g./hr.) Control	With inhibitor	% change
*25/4	Iodoacetate (5–35 min.)	1	33	10	− 70
	,, (45–115 min.)	1	14	0	−100
27/4	,,	1	128	11	− 91
	,,	0·1	79	11	− 86
	,,	0·017	130	62	− 52
25/5	*o*-Iodoxybenzoate	1	155	140	− 10
20/7	,,	10	164	18	− 89
	Washed tissue from above	0	158	9	− 94
30/8	*n*-Heptyl alcohol	*ca.* 12 (sat.)	222	14	− 93

* No added substrate.

higher concentrations. With the latter "retention" was shown to be negligible by tipping in 20 mg. 50 mM lactic acid at the end of the experiment. The effect was not reversed by washing the tissue (600 mg. wet wt. in 25 ml. glucose-$NaHCO_3$-Ringer) and resuspending in fresh fluid. It seems likely to be due to irreversible inactivation of some enzyme, probably not the lactic dehydrogenase since the redox-dye-catalysed O_2 uptake was less affected (Table 3, exp. 4/5).

Discussion

Although the above evidence is insufficient to establish the Embden-Meyerhof-Parnas scheme as a glycolytic mechanism for cartilage, it does suggest, especially in conjunction with the work of Lutwak-Mann [1940], the possibility of such a process. Pyruvate catalysis in particular suggests that it takes part in the oxido-reduction:

$$\text{Triosephosphate} + \text{pyruvate} = \text{phosphoglycerate} + \text{lactate}.$$

The dehydrogenase of cartilage for which Bywaters [1937] could suggest no function in the absence of oxidase evidently plays a part in this reaction, lactic and triosephosphate dehydrogenases being linked through a coenzyme. Iodoacetate inhibits at this stage, while fluoride prevents the rearrangement of phosphoglycerate to phosphopyruvate [Lohmann & Meyerhof, 1934] giving a fresh supply of pyruvate by dephosphorylation. In spite of the different sensitivities of autoglycolysis and glucolysis to pyruvate catalysis and fluoride inhibition, it is unnecessary to postulate separate mechanisms as did Bywaters [1937], since pyruvate concentration is evidently the limiting factor in attaining maximum glycolysis (cf. Dickens & Greville [1933] on rat testis and Jensen rat sarcoma). Fluoride inhibition is not complete except at high concentration and an inhibition of pyruvate re-formation insufficient to affect greatly the rate of autoglycolysis may yet affect maximal glucolysis, requiring a greater amount of catalyst. Lutwak-Mann [1940], working with calf cartilage, concluded that, in addition to the oxido-reduction mechanism, lactic acid could also be produced by

the action of cartilage glyoxalase, methylglyoxal being a normal intermediate in the breakdown of hexosediphosphate by the tissue. Since lactic acid production from hexosediphosphate was unaffected by 20 mM iodoacetate in her experiments, this mechanism cannot apply to old horse cartilage where almost complete inhibition of glycolysis was always observed with 1 mM iodoacetate both with and without added glucose. It seems likely that the glyoxalase mechanism is not specifically associated with cartilage metabolism but rather with the metabolism of all young and embryonic tissues, though glucolytic and glyoxalase enzymes react independently in chick embryos [Needham & Lehmann, 1937; Needham & Nowiński, 1937].

Summary

1. The O_2 uptake of horse articular cartilage, normally *ca.* 10μl./g./hr., is increased optimally by $3–10 \times 10^{-5}$ M methylene blue to 50–150 or by 10^{-4} M 2:4-dinitro-*o*-cresol to 20.

2. The cartilage from a joint showing arthritic changes failed to respond to dinitro-*o*-cresol.

3. The O_2 uptake of rabbit articular cartilage is similarly increased by 3×10^{-5} M methylene blue from 150 to 450μl./g./hr. and that of rabbit fibrous cartilage from the meniscus by 10^{-4} M methylene blue from 30 to 140.

4. The dye-catalysed O_2 uptake of horse cartilage is inhibited strongly by *n*-heptyl alcohol and partially by *o*-iodoxybenzoate. It is increased by HCN, sometimes increased by fluoride and unaffected by Ca^{++}.

5. The oxidation leads to the formation of pyruvate, but this is oxidized further with liberation of CO_2.

6. Pyruvate catalyses the glycolysis of cartilage and this catalysis is inhibited by fluoride.

7. The anaerobic glycolysis is also strongly inhibited by iodoacetate, iodoxybenzoate and *n*-heptyl alcohol.

I wish to thank Prof. E. C. Dodds for his interest and suggestions in connexion with this work, Dr E. G. L. Bywaters and Dr G. D. Greville for suggestions and technical advice in the early stages and the Mackenzie Mackinnon Trust of the Royal Colleges of Physicians of London and Surgeons of England for a research fellowship and for a grant to the Middlesex Hospital Medical School for the cost of the investigation.

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130. THE CONSTITUTION OF THE CAMBIUM, THE NEW WOOD AND THE MATURE SAPWOOD OF THE COMMON ASH, THE COMMON ELM AND THE SCOTCH PINE

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(*Received 10 May 1940*)

The composition of the mature cell wall of many plant tissues has been investigated in considerable detail, but there is little information as to the chemical nature of the wall in the earlier stages of its development. This is particularly so in woody plants in which the mature wood has been the subject of extensive research, while the study of the developing tissue has been almost completely neglected. Possibly one of the reasons for this neglect is the apparent difficulty of obtaining the necessary material. In the present work, it was found that the cambium and the differentiating xylem produced from it could be collected without great difficulty in amounts which permitted the determination of the main cell wall constituents.

There have been several previous analyses of the cambial sap of trees [Wislicenus, 1928; Schwalbe & Neumann, 1930; Wislicenus & Hempel, 1933] but this work was concerned with the water-soluble constituents of the sap and not with the insoluble matter of the cell wall. The cambial sap was obtained by removing the bark from the tree in early summer and scraping either the inner surface of the bark or the exposed surface of the wood. In the work of Schwalbe & Neumann [1930] and in the earlier work of Wislicenus [1928], the scraping was carried out by broken pieces of glass or porcelain. This method has the disadvantage of removing some of the tissues of the outermost sapwood, which together with the sap from the injured vessels are mixed with the tissues of the cambium and may give rise to misleading results. Even if the mature vessels are not ruptured, the so-called cambial sap will include differentiating xylem elements if collected from the surface of the xylem, and differentiating phloem elements if obtained from the inner surface of the bark. It is desirable that the two types of sap should not be mixed and in any work on the development of the wood only the cambium on the surface of the xylem should be investigated. Since the results are influenced to such an extent by the method of preparation, in investigations on the cambial sap the source of the sap should be clearly indicated. In his interesting work on the pectic substances of *Robinia*, Anderson [1936] dealt with the "cambium-phloem" layer, but from the description in the published paper it is not certain what tissues are included in this material.

In the present work, the material described as cambium includes a proportion of the true cambium and also the soft-walled differentiating xylem elements immediately within the cambium proper. The new wood comprises the xylem elements already produced from the cambium during the current year. For purposes of comparison the mature sapwood of the three species was also investigated.

Experimental

Methods

Material. The three species used included two hardwoods, namely the common ash, *Fraxinus elatior* L., and the common elm, *Ulmus sativa* Mill., and one softwood, the Scotch pine, *Pinus sylvestris* L. The material was collected from the hardwoods during the second week of May 1939, and from the Scotch pine, in which cambial activity is renewed at a later date, on 30 May. In each case well-grown trees were used.

Our method of obtaining the cambium differed only in detail from that employed by previous workers [Schwalbe & Neumann, 1930; Wislicenus & Hempel, 1933]. It is well known that with the resumption of cambial activity in spring, the bark readily "slips" from the woody core, the separation occurring at the cambial layer. The cambium and the differentiating xylem can then be scraped from the exposed surface of the wood. By using the rounded edge of a spoon to remove the soft developing tissue we avoided the inclusion of parts of the mature wood. After complete removal of the softer tissues it was found that the fully formed wood which had already been produced from the cambium during the current year could easily be separated from the late wood of the previous year with the aid of a slightly blunted chisel. In the ash there was a sharp transition between the gelatinous differentiating tissue and the more mature elements already produced; in the elm the distinction was not so marked, while in the pine the more uniform tissue could not be separated into cambium and new wood. The sapwood samples of each tree were obtained from the outer annual rings after removal of the cambium and the newly formed wood.

Treatment of fresh material. The cambium and the new wood of the three species were each dropped into boiling 95 % alcohol to give a final concentration of not less than 80 % alcohol. After refluxing for several hours, the alcohol was cooled and filtered off and the residue re-extracted with 80 % alcohol. The process was repeated until the extraction was complete. The extracted tissue was dried in the air and ground in a mortar to pass a 60-mesh sieve.

In each case the sapwood was air-dried and ground in a mill to pass a 60-mesh sieve. The powder was extracted with alcohol as for the cambium and new wood and again spread out to dry in the air.

Analytical methods

(*a*) *Water content.* The amount of water in the fresh material was determined by drying a sample in an electric oven at 105°. The amount of dry matter in the air-dried alcohol-extracted material was obtained in the same way.

(*b*) *Ash.* The ash content was determined by heating the residue from (*a*) in an electric muffle furnace at dull red heat.

(*c*) *Alcohol-soluble matter.* An aliquot of the 80 % alcohol extract was evaporated on a steam bath and drying completed in the oven at 105°.

(*d*) *Reducing substances.* After boiling off the alcohol, the reducing power of an aliquot of the alcohol extract was determined using the modifications adopted by van der Plank [1936].

(*e*) *Sucrose.* The hydrolysis of sucrose was effected by invertase.

All the following determinations were carried out on air-dried alcohol-extracted material, a correction being made for moisture content.

(*f*) *Nitrogen.* The total N was determined by the Kjeldahl method and expressed as protein after multiplication by the factor 6·25.

(*g*) *Cellulose.* Norman & Jenkins' [1933] modification of the Cross and Bevan process was adopted.

(*h*) *Lignin.* The material was digested with 72% H_2SO_4 for 16 hr., diluted to 3% and boiled for 2 hr. Although the product is not pure it can usually be taken to be a measure of the lignin content.

(*i*) *Furfuraldehyde.* The furfuraldehyde yield was determined by distillation with 12% HCl and precipitation as phloroglucide, using the modifications suggested by Angell *et al.* [1936]. Determinations were also carried out on the cellulose obtained in (*g*). The dried cambium cellulose could not be dispersed in the acid and in this case a fresh preparation was washed directly into the reaction flask.

(*j*) *Uronic acid.* The procedure of Dickson *et al.* [1930] was followed. Determinations were carried out before and after extraction with 0·5% ammonium oxalate, the loss in uronic acid after extraction yielding a value for the total pectin content of the material.

(*k*) *Mannan.* Determinations of mannan were restricted to the softwood material. Nowotnowna's [1936] method was employed.

Results

The results of determinations on the fresh material and on the alcohol extract are set out in Table 1, while Table 2 contains the results of determinations on the alcohol-extracted material.

Table 1. *Compositions of cambium, newly formed wood and mature sapwood of common ash, common elm and Scotch pine*

	Water as % fresh material	Dry matter as % fresh material	Ash as % dry matter	Alcohol-extractable material as % dry matter	Reducing substances as % dry matter	Sucrose as % dry matter
Ash:						
Cambium	88·5	11·5	10·4	74·7	3·22	26·8
New wood	74·3	25·7	3·85	27·6	1·73	6·92
Sapwood	34·7	65·3	0·49	5·83	0·26	1·35
Elm:						
Cambium	90·55	9·45	20·1	47·7	2·27	11·2
New wood	81·2	18·8	7·27	29·0	2·16	1·66
Sapwood	47·8	52·2	0·48	5·66	0·59	0·21
Pine:						
Cambium	86·0	14·0	6·11	62·0	10·2	27·2
Sapwood	48·5	51·5	0·22	3·20	0·41	0·32

In each species the water content of the cambium is high, as would be expected in young actively growing tissue. The new wood of ash and elm has a much higher water content than the older sapwood.

The ash contents of the three cambium samples are high when expressed as percentages of the dry matter, but form only small proportions of the fresh weights. The amount of mineral matter in the old wood is low, while that of the new wood lies between the figures for the cambium and old wood.

In the cambium of all three species the alcohol-soluble part forms a high proportion of the dry matter, especially in ash. This fraction is only small in the old sapwood, but exceeds 25% of the dry weight in the newly formed wood.

The free reducing substances formed only a comparatively small proportion of the alcohol extract of the cambium, which had, however, a much higher

sucrose content. Klason [1929] also found a high concentration of sucrose in the cambial sap of the fir, while Wislicenus [1928; Wislicenus & Hempel, 1933] states that invert sugar forms the main part of the dissolved substance. The latter result could be obtained by the action of an enzyme during the preparation of an aqueous extract of the sap. In the present work activity of enzymes was avoided by dropping the freshly collected cambium into boiling alcohol. The sugar content of the new wood is much lower than that of the cambium, but it should be pointed out that some of the sugar in the cambium has possibly escaped from the vessels of the new wood. As might be expected, there is little sugar in the older sapwood.

Since we were mainly interested in the cell-wall substances no determinations were made of the other components of the alcohol extract, which also includes glucosides, lipins, soluble N etc.

The residue from the alcohol extraction contains the main constituents of the cell wall and was analysed in greater detail than the alcohol extract.

Tests for starch with iodine solution were negative for all samples of the cambium and new wood; the older sapwoods of elm and pine gave only slight colorations, while a strong starch reaction was obtained in ash sapwood. These results were to be expected since it is not likely that starch would accumulate in young actively growing tissue.

Since all the cells of the cambium region have living contents, the high N obtained for the cambium is in accordance with expectation. The new wood has a higher N content than the older sapwood.

The percentage of cellulose is considerably lower in the cambium than in the new wood or sapwood. In both ash and elm the cellulose content of the new wood is less than that of the older sapwood. The lower value of the new wood may be in part a result of incomplete development, or may be explained wholly by the difference in composition known to exist between the early and late woods of the single annual ring [Ritter & Fleck, 1926; Preston & Allsopp, 1939]. The cellulose obtained from the new wood and older sapwood was white and fibrous, while that of the cambium was grey and horny. A specimen of the cellulose from ash cambium was examined by Dr R. D. Preston, of this Department, who found that the X-ray diffraction pattern was closely similar to that of cotton cellulose.

The furfuraldehyde produced from the cellulose arises from the cellulosans which in the hardwoods are built up almost entirely from xylose [Norman, 1937, 1, p. 20]. In ash and elm the xylan content of the cambium cellulose is greater than that of the sapwood, but less than that of the newly formed wood. The high proportion of xylan in the cambium cellulose is of interest in suggesting that the xylan arises early in the life of the cell, and is probably not secondarily produced from the cellulose. The lower xylan content of the sapwood as compared with the new wood may again be explained by the fall in pentosan content from early to late wood [Ritter & Fleck, 1926; Preston & Allsopp, 1939], but in the ash the difference is of such magnitude as to suggest that other factors might operate, as for example, a change in composition subsequent to vessel differentiation. The high xylan figure of the ash new wood is in excess of the value previously recorded for other wood celluloses. The mature sapwood of pine like that of the other species has a lower xylan content than the cambium. In addition to xylan, mannan is found in the cellulose of conifers. The proportion is surprisingly high in the cambium of pine and is far in excess of the amount in the sapwood cellulose.

The lignin values for the mature woods of the three trees merely confirm the previous analyses of this material. The new woods of ash and elm are again of

interest in the possession of higher lignin figures than the sapwood. In this case it is almost certain that the difference reflects that normally observed between early and late woods. Ash cambium which was the purest of the three species has a very low lignin content, the elm cambium has a higher lignin content, part of which is certainly derived from small amounts of the new wood present as impurity, while the pine cambium has a relatively low lignin content. Although the lignin values for the differentiated wood are reasonably accurate, there are probably fairly large errors in the determinations on the cambium, where there is a high concentration of substances such as proteins, pentoses etc. which increase the yield of apparent lignin [Norman, 1937, 2]. But although the figures are not precise they do indicate that the material used in the present investigation consists of relatively unlignified tissue.

The preparation of the cellulose by Norman & Jenkins' method [1933] supplied further information of the nature of the lignin. It is well known that the lignin of gymnosperm woods differs from that of angiosperm woods in yielding a brownish colour instead of magenta when warmed with sodium sulphite solution after treatment with chlorine. In the present work we were able to extend this observation to the cambium of the species investigated. Following two hypochlorite treatments the usual treatment with chlorine and sodium sulphite resulted in a magenta colour with ash and elm cambium and a brown colour with that of pine. This treatment removed all the lignin from the cambium and no coloration was produced after a second chlorination.

The newly differentiated woods gave higher lignin values than the mature wood, but it would seem that the polymerization of the lignin units is of a lower grade in the former, since the new woods of ash and elm were delignified after 4 chlorinations while the mature woods required 7 and 9 chlorinations respectively. Pine wood required 8 chlorine treatments.

The pectin values given in Table 2 are obtained by multiplying the fall in CO_2 output after extraction with 0·5 % ammonium oxalate by the factor 5·66.

Table 2. *The compositions of the cambium, newly-formed wood and mature sapwood of common ash, common elm and Scotch pine*

	Ash			Elm			Pine	
Material ...	Cambium	New wood	Sapwood	Cambium	New wood	Sapwood	Cambium	Sapwood
Cellulose	20·2	51·9	58·3	20·7	53·0	57·5	25·1	61·8
Lignin	4·60	23·2	20·9	13·5	25·5	24·8	8·56	26·1
Pectin	21·6	3·85	1·58	9·91	3·72	1·68	16·6	0·96
Protein	29·4	5·56	1·37	30·0	5·19	1·73	20·8	0·83
Total furfuraldehyde	10·6	17·3	15·2	10·3	15·1	14·2	13·0	6·42
Furfuraldehyde not in cellulose	7·04	6·10	6·16	6·64	5·00	5·52	10·67	3·49
Xylan in cellulose as % cellulose	26·9	33·7	24·5	26·3	29·8	23·8	15·0	7·9
Mannan in cellulose as % cellulose	—	—	—	—	—	—	21.9	6·16
Total uronic anhydride	20·2	8·20	5·25	12·5	7·86	6·44	15.7	3·71
Uronic anhydride not extracted by ammonium oxalate	4·90	5·48	4·10	4·6	5·23	5·25	4·00	3·03
Furfuraldehyde in hemicellulose	1·66	4·13	4·95	3·66	3·12	4·04	6·48	2·64

The high values recorded for ash and pine cambium are very striking, while elm cambium has also a relatively high pectin content. It is clear that the wood contains at most only a small amount of pectin, especially in pine. The quantity in the new wood is still low but higher than that of the mature wood.

Our results are in general agreement with the more detailed investigations on the pectic substances of woody materials by Anderson and his co-workers [1936; 1937]. These workers also find that conifer wood has a lower pectin

content than hardwoods, while their "cambium-phloem" had a high pectin content, although the values did not attain the magnitude of those obtained in the present work.

At the present time there is unfortunately no satisfactory method of determining hemicelluloses, but a consideration of the furfuraldehyde and uronic anhydride contents affords some information as to the nature and amount of this fraction. The non-cellulosic furfuraldehyde arises from the pentoses and uronic acids contained in pectin and in the hemicelluloses. The furfuraldehyde yield of the uronic acids is known [Angell *et al.* 1936] and many analyses of pectin preparations have shown that the furfuraldehyde yield is never far from 20% [Norman, 1937, 1, p. 80]. Since the pectin and the hemicellulose uronic anhydride have been determined, subtraction of the calculated furfuraldehyde yield of these constituents from the total non-cellulosic furfuraldehyde affords an approximate measure of the furfuraldehyde produced from the pentosans of the hemicelluloses. The figures obtained in this way are set out in Table 2 as "furfuraldehyde in hemicellulose". It should be mentioned that in our calculations we have made the assumption that we are concerned only with one uronic acid. The error involved is considerable, but like that arising from the fact that the furfuraldehyde yield of a mixture is not the sum of the furfuraldehyde yields of the constituents [Angell *et al.* 1936] is not sufficiently great to affect the general conclusions. The differences are enough to show that ash cambium has a far lower pentose content than either the new or mature wood. This is not the case for the less pure cambium of elm, but the other analyses for elm cambium are also characteristic of a more mature material than those of ash cambium. The pine is clearly quite a different case, the cambium containing far more pentose than the mature wood. It would seem that in ash and elm the differences between new and mature woods are sufficiently great to warrant the statement that the mature wood has a higher pentose content than the newly formed wood. The results show a higher uronic acid:pentose ratio than that obtained in isolated hemicellulose preparations [Norman, 1937, 1, p. 40]. This discrepancy can be explained by a failure to remove all the pectin from our samples before determination of hemicellulose uronic anhydride. It is also possible that the lower ratio in isolated hemicelluloses might be accounted for by degradation of uronic acids during extraction.

Discussion

The analytical results described above reveal that the cambium and differentiating xylem of ash, elm and pine have similarities in the composition of the cell wall with other young tissues. The high pectin content is particularly characteristic of the younger stages of the cell wall [Buston, 1935; Griffioen, 1938; 1939]. It is in this respect, and also in the lower lignin:cellulose ratio that the cambium and differentiating elements differ chiefly from the mature wood. The analyses of the newly formed wood supplied some evidence that even after vessel differentiation small changes in composition take place involving a further loss of pectin, a small increase in the amount of encrusting pentosans and an increase in the resistance of the lignin components.

Several hypotheses of the origin of the constituents of mature tissues have been based on a consideration of the composition of younger tissues. A fall in the proportion of one component accompanied by an increase in that of another has been cited as evidence of the production of the second from the first substance. In this manner, the great fall in concentration of pectin which occurs during the development of lignified tissues has been taken to indicate that the lignin is formed from the pectic constituents [Griffioen, 1938; 1939; O'Dwyer, 1932]. Our

results might be claimed to support this view, but it should be emphasized that the change in the concentration of pectin in passing from the cambium to the mature wood is no evidence of an alteration in the total quantity. During the development of the xylem elements the thin walls of the differentiating cells undergo a great increase in thickness and it is probable that little or no pectin is deposited in the additional thickening layers. If the pectin present in the younger stages persisted unchanged, the total amount would undergo no alteration while the concentration would be greatly reduced. This view is supported by the work of Buston [1935] who found no fall in the pectin content during the lignification of rose shoots, although the concentration was considerably reduced. Even if the pectin were reduced in amount it would not necessarily imply that it had undergone transformation into another cell wall constituent.

In the ash cambium a large amount of pectin is accompanied by a small amount of encrusting pentosan. Such evidence is used in support of the view that the encrusting hemicelluloses arise from pectins, but the change in proportion of these constituents can equally readily be explained by an alteration in the composition of the later thickening layers of the cell wall. Several workers have suggested that the encrusting pentosans afford a source of lignin and in this connexion it may be of some significance that in the cambium of pine the pentosan content is unusually high but is quite low in the mature wood. More detailed work on the composition of the hemicelluloses of cambium is desirable and might throw further light on the origin and subsequent fate of these comparatively neglected cell-wall constituents.

SUMMARY

The woods of the common ash, common elm and Scotch pine were divided into three fractions: cambium + differentiating xylem, newly formed wood and mature sapwood. Analyses were made of each fraction.

The composition of the cambium was found to be similar to that of other differentiating tissues especially in the high pectin and relatively low lignin contents. There is some evidence that further slight changes occur in the composition of the wall after vessel differentiation is complete.

The writers wish to thank Prof. J. H. Priestley for affording them every convenience during the progress of this work. They are indebted to Dr R. D. Preston for helpful suggestions and assistance in the collection of the material.

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131. THE PHYSIOLOGICAL ACTION OF ABNORMALLY HIGH TEMPERATURES ON POIKILOTHERMIC ANIMALS

1. TEMPERATURE ADAPTATION AND THE DEGREE OF SATURATION OF THE PHOSPHATIDES

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(*Received 23 May 1940*)

At temperatures in the region of 40–45° most animals die within a few hours. This applies equally to hot- and to cold-blooded animals. Often, however, temperatures lethal to cold-blooded animals are considerably lower than that limit. The cause of death by heat and the mechanism of heat injury at temperatures below those where a visible destruction of the tissues (e.g. coagulation of protein) takes place have given rise to a considerable amount of speculation and numerous hypotheses have been advanced to explain the phenomena observed.

In the older literature on the subject death by heat was attributed mostly to the coagulation of proteins; more recently other hypotheses [recently reviewed by Belehradek, 1935] have been put forward, such as destruction of enzymes, asphyxiation or other disturbance in the equilibrium of protoplasm through accumulation of waste products. While none of these hypotheses is yet proved experimentally the one most deserving further investigation is the "lipoid liberation theory" since it is the only one where evidence has been provided linking the phenomenon of heat adaptation—i.e. the capability of an organism to increase its heat resistance—with data on the constitution of an important component of the protoplasm.

It has been shown by various authors that the melting point of fats of micro-organisms and of higher plants and animals varies with the temperature at which they have been laid down. When examined at a given temperature fat formed at a higher temperature is found to be more solid than fat formed at a lower temperature. Since the melting point of a fat depends largely on its degree of saturation, the iodine value (number) of a fat as a measure of double bonds in the fatty acid chains is a good measure for its melting point. Thus the iodine number of the visceral fat of a mammal is lower than that of the subcutaneous fat. The number is also lower in oils from tropical plants than from plants grown in a cooler climate. (Numerous other examples in Belehradek [1935].)

Various workers observed changes occurring in the lipoid constituents of the cell on heating. Fauré-Fremiet [1924] found in the eggs of *Sabellaria* granules appearing on heating which seemed to be of lipoid character. A similar "liberation" of lipins was found by Heilbrunn [1924] who centrifuged eggs of *Arbacia* after heat treatment. Koeppe [1903] and Rywosch [1911] attributed haemolysis of erythrocytes to a melting of the superficial lipoid membrane. Heilbrunn [1924] first drew special attention to the correlation between heat resistance and the

melting point of protoplasmic fats, a point which has later been enlarged upon by Belehradek [1931]. The latter, formulating the "lipoid liberation theory", links up the heat "adaptability" of the protoplasmic fats, as expressed by the correlation between the melting points—degree of unsaturation—of fats and the temperature of their formation, with the adaptability of the whole organism to high temperatures. According to him heat injury, whether reversible or irreversible, is caused by the melting of lipoid constituents in the cell or in the cellular membranes. Fat formed at a higher temperature and consequently of a higher melting point would render an organism more resistant to the damaging effect of high temperatures.

As already mentioned, there are well-established examples available about the comparative degree of saturation of lipins found in plants and animals from different climates or of different habitat. The literature contains only a few, but well-established examples in which changes in the iodine number of fats have been demonstrated by growing an organism at different temperatures. [Terroine *et al.* 1927; 1930] on bacteria, Pearson & Raper [1927] on *Aspergillus.*] Although the phenomenon of adaptability of the living organism to the temperature of its environment has been regarded almost as an axiom by ecologists, it is supported by surprisingly few certain examples in the literature. The most striking examples of temperature adaptation are found in the work of Hathaway [1927], Wells [1935, 1, 2], and Sumner & Doudoroff [1938], all relating to fishes. The phenomena of heat adaptation on the one hand and changing melting point of lipins on the other have, however, never been investigated in the same organism and their combination in the lipoid liberation theory is based on mere analogy.

The object of our work is to fill this gap in our knowledge by breeding an organism at different temperatures and subsequently testing for differences in its heat resistance and in the saturation degree of lipins of individuals bred under otherwise comparable conditions at different temperatures.

Adaptation to high temperatures

The larvae of two species of flesh flies, *Calliphora erythrocephala* Meigen and *Phormia terra-novae* R.D., differ widely in their tolerance of high and low temperatures. *Calliphora* develops from the egg to the fully grown larva at any temperature between 12 and 31°, the closely related *Phormia* only develops at temperatures between 18 and 36°. To attain full growth the larvae of both species require about 3 days at the higher and 16 days at the lower end of the respective ranges. The larvae, thus bred at different temperatures to their full size, were submitted simultaneously to a test for their resistance to a constant high temperature. The method adopted was similar to that described by Mansbridge [1936]. The larvae were dropped suddenly into the temperature required and the time after which approximately 50% of them were still undamaged, i.e. would pupate and develop to normal flies, was determined. The following tables present the summary of the experiments.

Table 1. *Heat resistance of* Phormia

	Period in hr. after which 50% were killed	
Tested at	(Reared at 18°)	(Reared at 36°)
46°	$\frac{1}{2}$–$\frac{3}{4}$	$2\frac{1}{2}$
45	$3\frac{1}{2}$	>7, <15
44	7–11, 15	15, 18
43	19	24

Table 2. *Heat resistance of* Calliphora

Tested at	Period in hr. after which 50 % were killed (Reared at 12°)	(Reared at 30°)
41°	1	1½
40	1½	2½
39	3	5
38	>4 <10	13

From the figures in Tables 1 and 2 it is clear that larvae which were bred at a higher temperature could resist a given high temperature for a considerably longer period than larvae which were bred at a lower temperature. Or, considering the temperature which heat-adapted larvae and non-adapted specimens could tolerate for a given time, it appears that the difference is approximately 1°. Thus, for *Phormia*, reared at 36°, a temperature of 46° for 2½ hr. has about the same effect as a temperature of 45° for the same period for larvae bred at 18°.

The correlation between the iodine values of the fatty acids of the phosphatides and the temperature of breeding

For these experiments the same species of larvae were bred under identical conditions as in the heat resistance experiments described.

It had been proposed at first to work with the total fats of the body for these investigations. This, however, was abandoned for the following reasons. The work of Evans [1932] has shown a selective utilization of the reserve fats in blowfly larvae in the period preceding pupation, when the insects have stopped feeding (so-called prepupae), and it was feared that this selective utilization would constitute a considerable source of error, as these larvae are at that stage of their life history entirely dependent upon the reserve materials of their body. This source of error would be especially large, as the known difference in duration of the life history at different temperatures makes it evident that, for instance, 1 hr. at 36° does not at all correspond to 1 hr. at 18°, and that even the smallest differences in "physiological" age would greatly add to the possible error.

The phosphatides, on the other hand, are now generally considered as the "vital" fats, as opposed to the reserve fats. Though very little is known about their function so far, we do know that they occur in every cell and that their presence is apparently essential to the continued existence of protoplasm. It is therefore improbable that they would be utilized as reserve fats, though a certain proportion might always be found in a transit stage engaged in metabolic processes. But, whatever their alleged "vital" role may be, it is most likely that they would be involved in the protoplasmic processes connected with heat injury.

In the method of analysis the procedure of Terroine *et al.* [1930] was followed with certain modifications.

At least 500 fully grown larvae, with their guts empty, were extracted for 48 hr. in a Soxhlet apparatus with absolute alcohol. From the extract obtained, the alcohol was evaporated off, and the residue extracted several times with ether of sp.gr. 0·720, to which, after concentration, 4 vol. acetone were added. The resulting precipitate was allowed to stand for 15 min., centrifuged, the liquid decanted off, and then redissolved with ether. The precipitation was repeated four times in the early part of the work, when it was assumed that the material thus obtained would be fairly pure phosphatide, or at least phosphatide of a comparable standard of purity. The results of the determinations obtained with

material prepared by this method are given in Table 3, under the heading "Iodine values by first method".

A comparatively large divergence in the properties of material thus obtained, led to an investigation of its purity, and it was found that the phosphorus/nitrogen ratio still varied considerably in materials prepared in the above way. It was therefore decided to work in future on material showing a constant percentage of phosphorus, and to continue precipitation until this was obtained.

Phosphorus was estimated by the colorimetric method of Martland & Robison [1926], measuring the total phosphorus as inorganic phosphate to within $\pm 0{\cdot}005$ mg.

The results for the highest degree of purity obtainable were as follows:

	Theoretical value, pure lecithin + kephalin %	Actual value %
Phosphorus	4·8	3·4–3·6
Nitrogen	2·4	2·8–3·1

The material finally worked with was therefore by no means pure phosphatide, but was in all probability still adulterated with protein or urea. However, the degree of purity obtained need not be regarded as unsatisfactory, as commercial technical lecithin, which was tested for comparison, only contained about 2·8% phosphorus.

For the second series of estimations (called "second method" in Table 3), the phosphorus in the acetone precipitation was estimated and precipitation repeated until the phosphorus percentage remained constant at the above value (approx. 3·5%). The "phosphatide" thus obtained was then saponified with 10% alcoholic KOH on a boiling water bath under reflux.

The further procedure was then as used by Evans [1932] and Rainey [1938] in their work on blow-fly larvae. Saponification was carried out for 11 hr. at least, after which time most of the alcohol was boiled off under reduced pressure and the resultant syrupy solution was taken up with a little water, from which the unsaponifiable matter was removed by shaking five times with ether in a separating funnel. From the ethereal solution thus obtained any remaining soaps were shaken out with water, which was united with the original soap solution. From this the fatty acids were precipitated by adding hydrochloric acid (1 part of conc. HCl to one part of water). These were removed by shaking out five times with ether. The solvent was removed, the residue, which still contained a certain amount of impurity, was taken up with light petroleum (B.P. 40–60°), and the fatty acids recovered by evaporation of the solvent in a weighed flask. The iodine values were measured by the method described by Dam [1924], and the results are as follows:

Table 3. *Iodine values of phosphatides*

Species	Temp. of breeding	Iodine values by First method	Second method
Phormia	18°	88, 89, 92, 86	91, 92
	27	64·5, 84, 76, 78	71, 72, 73·5
	36	69, 60·5, 65·8, 66·7	69, 62, 63
Calliphora	12	78, 88, 89, 88, 82·7, 84, 85	—
	15	—	96, 95·8
	27	72, 76, 72·5, 84, 83, 75·5	72·7, 75
	30–31	65·2, 66·2, 70	70, 70, 67

The results obtained by the "first method" show more scatter than those from determination of phosphatides with constant phosphorus content, but there is no significant difference between the two sets.

The results are graphically represented in Fig. 1. It is apparent that the degree of saturation of the phosphatides is directly dependent upon the tem-

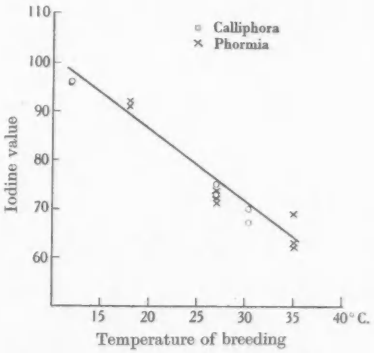

Fig. 1. The relation of the iodine values of the phosphatides of the larvae of *Calliphora* and *Phormia* with the temperature of breeding.

perature of breeding in the two closely allied species investigated. The fatty acids of the phosphatides laid down at the higher temperature in both species are more saturated than those produced at lower temperatures, proved by a lower iodine value. The difference for the extreme temperatures of breeding, a temperature difference of about 18°, is about 26 units in each species.

Discussion

It is shown that blow-fly larvae, reared at higher temperatures, not only become more resistant to high temperatures than larvae reared at a lower temperature, but that the melting point of their cellular fats is also "adapted". In both species of insects the range of change of the iodine value is about equal. The mean difference between the values at the highest and lowest temperatures of breeding in *Phormia* is about 26 units, and in *Calliphora* also 26 units, with a range of breeding temperature of 18°, in both cases. This amount of adaptation may appear rather high, but the values are well in agreement with those found by other workers. Pearson & Raper [1927], working on the total fat of two species of fungus, found the mean iodine value of *Aspergillus niger* to be 149 if bred at 18°, 129 at 25° and 95 if the cultures were kept at 35°. In *Rhizopus nigricans* the corresponding figures were 88 for 12° and 78 for 25°. Terroine *et al.* [1930] found that the micro-organism *Sterigmatocystis nigra* in cultures grown at 18° had an iodine value of 135 for the fatty acids of the phosphatides, and the same organism grown at 38 gave a mean of 99. "Bacille de la Fleole" gave mean values of 51 and 31 for cultures bred at 18 and 38° respectively. The values for the two species of fly larvae can therefore be regarded as well within the range to be expected from other work.

No figures are available for the degree of saturation of fatty acids of the phosphatides in other insects. Ackerman [1926] cultivated the aphid *Rhopalosiphum prunifolia* at different temperatures and found that the solidification

point of fatty globules in the body fluid increased with the temperature of cultivation. Rainey [1938] reared the larva of another flesh fly, *Lucilia sericata*, which is closely related to the species used by us, at temperatures of 15, 25 and 35° and found the iodine values of the total fatty acids to be 75·8, 72·2 and 71·0 respectively. These figures, although showing a clear tendency to falling iodine number with increasing temperature, are not regarded as significant by Rainey, the difference in iodine value being just outside the experimental error and the larvae having been used for the determinations at a different physiological age. As the physiological role of the phosphatides is so different from that of the greater part of the total fat, which constitutes storage material, no conclusions can be drawn from a comparison of Rainey's figures and our own.

The many data now available of which those quoted above constitute only a fraction makes it clear beyond doubt that the rule that the constitution of fats depends on the temperature at which they are laid down applies to all living matter. Leathes & Raper [1925] and Pearson & Raper [1927] point out that lipoid substances are formed not by a single chemical process but by a catenary series of changes. The unsaturated compounds are formed first, and the saturated ones arise in turn from these. At low temperature this process cannot be completed but stops at a higher degree of unsaturation than would have been the case if the formation had taken place at a higher temperature.

Table 3 and Fig. 1 show that the mean iodine values for the two flies used by us vary only according to temperature and not to species within the same range. However, it is evident that this is not applicable to all living matter and that completely different sets of results are obtained with different sorts of organisms. This is shown clearly by comparing our results with those of Terroine, quoted above. Whether the similarity in the two closely related insects examined is merely accidental or whether phylogenetic relationships will also lead to a similarity in this respect is a question demanding further investigation. That fats derived directly from the food are often laid down unchanged as reserve material is a well-known fact in vertebrates. Yuill & Craig showed [1937] that larvae of *Lucilia sericata* reared on different diets showed a difference in the iodine value of their fats according to the food offered to them. However, as the fly larvae in our own experiments were grown at different temperatures on the same food (lean meat), the differences found in the constitution of the phosphatides cannot be due to the food. It is generally assumed that phosphatides, e.g. lecithin, are not obtained directly from the food material in unchanged form but are products of synthesis within the body itself.

The results obtained for *Phormia* and *Calliphora* support the lipoid liberation theory in so far that for the first time there has been found a direct correlation between the saturation degree of the phosphatides and the heat resistance of the whole organism. However, the results obtained make it very unlikely that death at moderately high temperatures is due simply to a melting of the protoplasmic fats. Otherwise there would be no explanation of the fact that the lowest temperature which each of the two species of fly larvae can tolerate for a given period is approximately 7 degrees lower for *Calliphora* than for *Phormia*. Adaptation consists only of pushing the lowest lethal temperature for a given period up by approximately 1 degree. *Calliphora* and *Phormia*, bred at 27°, are killed at an exposure of 5 hr. at 39 and 45° respectively and yet the iodine values of the phosphatides of both species are the same, namely 75.

From this the conclusion may be drawn that it is unlikely that a breakdown of lipins in a purely physical way, causing for instance a change in cell permeability, is the cause of heat injury at the comparatively low temperatures

required. The lipoid liberation theory of adaptation to high temperatures, while certainly substantiated by the present work in so far as such striking differences in the constitution of lipins bear out the predictions made by it, cannot explain fully and satisfactorily the phenomena of heat injury and heat adaptation. Other factors must play their role, but the striking adaptation of the saturation degree of lipins described in this work leads to the tentative suggestion that the physical nature of these fats may have a decisive influence on the chains of physiological processes involved.

Summary

The adaptability to high temperature and the nature of the phosphatides have been studied in fully grown larvae of the blow-flies *Calliphora erythrocephala* Meig. and *Phormia terra-novae* R.D.

The results are as follows:

1. In both species of insect the amount of heat adaptation is such that larvae bred 18° higher than others will be able to withstand the injurious effects of high temperature for one degree more for the same time of exposure than those bred at the lower end of the temperature range.

2. The degree of unsaturation of the phosphatides of these larvae, measured by their iodine values, is dependent upon the temperature of breeding only, and was found to show a difference of 26 units in both species for a difference in the breeding temperature of 18°.

3. These results are discussed in the light of the "lipoid liberation theory", as advanced by Heilbrunn and Belehradek. While the strong adaptation of the phosphatides to the temperature at which they have been laid down suggests a role for these substances in the mechanism of heat adaptation, the fact that two closely allied species of insects, bred at the same temperature and having the same iodine value for the fatty acids of their phosphatides have different resistances to heat, shows that a physical breakdown of the fatty substances cannot be the direct cause of heat injury.

The authors are greatly indebted to the Agricultural Research Council for a grant defraying the costs of this investigation, and to Profs. J. W. Munro and A. C. Chibnall who provided the facilities for the work in the departments of Zoology and Applied Entomology and Biochemistry respectively; they also wish to thank the above named and Dr H. W. Buston for much valuable advice and suggestions.

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132. STUDIES ON THE SECRETION OF MILK FAT

3. THE EFFECT OF THYROXINE ADMINISTRATION ON THE BLOOD LIPOIDS AND ON THE NATURE OF THE MILK FAT

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(*Received 23 May 1940*)

In planning experiments to investigate the physiological and biochemical processes involved in the secretion of milk fat, one of the main objects has been to find whether a relation exists between the lipoids of the blood on the one hand and the fat of the milk on the other. In the course of the work, however, much difficulty has been experienced in finding some means by which alterations in the nature and level of the blood lipoids could readily be brought about, for in the ruminant these particular constituents change but slightly even over relatively long periods.

A study of the literature suggested that thyroxine might possibly be of value for this purpose. That the administration of this hormone causes a large increase in the secretion of milk and milk fat has been shown by Graham [1934, 1, 2], Jack & Bechdel [1935], Folley & White [1936] and Herman *et al.* [1938], but in none of the experiments so far carried out with ruminants has an analysis been made of the changes which may occur in the various lipoid fractions of the blood. It is generally known that in man and in many other animals hyperthyroidism is accompanied by a decrease in the concentration of blood cholesterol and other lipoids, but it appears that this effect is not common to all species. The only reference to the effect of thyroxine on the blood lipoids of the lactating cow is that of Graham *et al.* [1937] who cite some previous but unpublished work of Graham & Jones carried out in 1935. They report that thyroxine brought about an increase in blood sugar level and a decrease in the total fatty acids of the arterial blood, but it is evident that no study was made of the different individual fractions of the blood lipoids. It is important that the individual fractions should be considered as separate entities in connexion with lactation, for it is quite possible that a fall in the total fatty acids of the blood might be accompanied by an actual rise in the triglyceride fraction, which forms a very small proportion of the total blood lipoids and has been shown to be the main precursor of milk fat. That the triglycerides of human blood plasma may not be affected while the other lipoids decrease during hyperthyroidism has been shown by Boyd & Connell [1937]. It was therefore decided to investigate this point in greater detail by administering thyroxine to lactating cows and analysing as extensively as possible the various fractions of the blood lipoids before, during and after the enhancement of the milk yield which, on the basis of previous work, the hormone would be expected to bring about.

At the same time another aspect of the subject, i.e. the composition of the milk fat, was also investigated. If in the future it should become possible to secure greater milk production by breeding animals possessing greater thyroid activity, the question naturally arises as to whether thyroxine is likely to cause any change in the nature of the milk fat when it brings about increases in its

yield. Only one publication has so far appeared in which the analysis of a milk fat secreted by cows receiving thyroxine has been mentioned. In this work Hilditch & Paul [1936] used the fat from such cows for a study of the minor fatty acid components of milk fat, but since these authors were not primarily interested in the effect of thyroxine on the general nature of the fat, no comparison was made between its composition before, during and after hormone treatment. The analytical figures for the fat used by Hilditch & Paul fell within the usual normal limits, but since the extremes within which milk fat may vary and yet remain "normal" are wide, it was decided to examine this point in more detail.

There were therefore two main objects in the present work: first, to examine as far as possible the changes brought about by thyroxine in the amount and nature of the blood lipoids; and second, to study the quality of the milk fat before, during and after the enhanced yield which the hormone is known to effect. At the same time the amounts of non-fatty solids, protein and lactose in the milk were determined in order to collect further evidence on the effect of thyroxine on the concentrations of these particular milk constituents.

Experimental

The particulars of the 4 animals used are given in Table 1, where it will be seen that the first 3 cows were treated with thyroxine under autumn conditions and the fourth in the summer. All the animals were allowed free access to pasture and were given a small ration of crushed oats and maize twice daily when brought in for milking. Composite samples of the morning and evening milk were taken every day for analysis, and small representative samples of the fat were prepared as described in the previous paper of the present series by Smith & Dastur [1938]. Blood samples were also taken at suitable intervals from the subcutaneous mammary veins.

Table 1. *Particulars of the animals used*

	Age years	No. of lactations	Weeks in milk	Time when exp. was made
Cow 1	4	2	27	Sept.–Oct. 1938
2	7	5	34	
3	6½	4	37	
Control	6	3	28	
Control	10	7	34	
Cow 4	11	8	15	May–July 1939
Control	7½	5	14	

The thyroxine was administered as a solution made by dissolving thyroxine sodium B.P. (British Drug Houses, Ltd.) in dilute NaOH and neutralizing the excess of alkali with dilute HCl until the first trace of precipitate appeared. The mixture was then made up to such a volume that 10 ml. contained 10 mg. of the hormone. 10 ml. of this preparation were injected intramuscularly into the thigh once each day during the experimental period. This treatment was continued for 10 days with Cows 1, 2 and 3 and for 15 days with Cow 4, the experimental periods being preceded and followed by control periods in which no hormone was given. In order to be sure that any marked variation in milk yield observed during the experimental period was actually due to the treatment, a record was made of the milk yields of control cows at approximately the same stage in lactation and kept under identical conditions except that they did not receive the hormone. It will

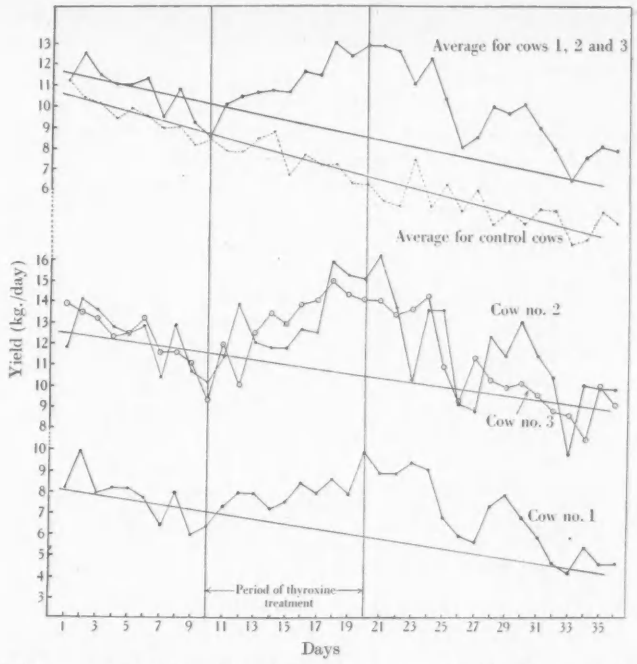

Fig. 1. The milk yields of Cows 1, 2 and 3 and the average milk yield of two control cows.

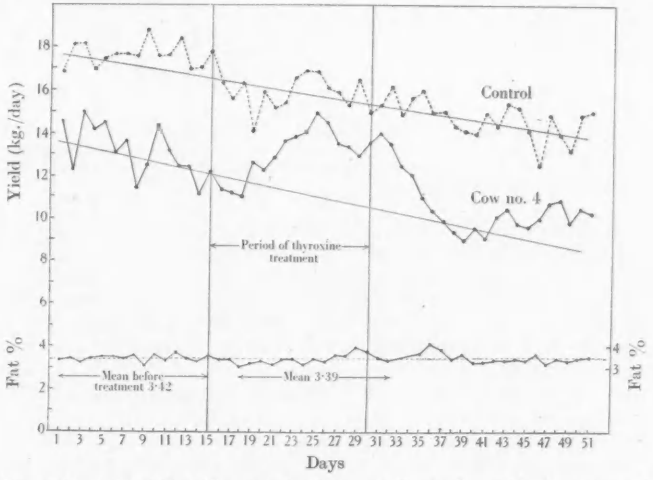

Fig. 2. The milk yields of Cow 4 and of the control animal, and the fat content of the milk secreted by Cow 4.

be seen from Figs. 1 and 2 that, unlike those of the controls, the milk yields of the experimental cows were much enhanced as a result of the thyroxine administration.

Milk and milk fat analysis

The milk samples were analysed for total solids [Golding, 1934], fat (Gerber), lactose (polarimetrically) and protein (Kjeldahl, total N × 6·38). The results for Cow 4 and the average values for Cows 1, 2 and 3 are recorded diagrammatically in Figs. 2–4 and will be discussed more fully later.

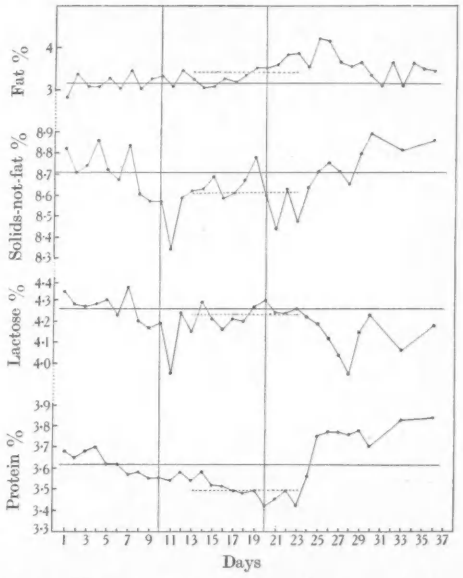

Fig. 3. The concentration of various milk constituents. Mean values for Cows 1, 2 and 3 (whole milk). (The straight continuous line shows the average for the pre-thyroxine period. The straight broken line shows the average from the 3rd day after thyroxine treatment began until 3 days after it ceased.)

Daily alterations in the composition of the milk fat were followed by determining the Reichert Meissl and iodine values, which are shown in Fig. 5. In order that the fat sample required should not be too large, the Reichert Meissl values were all estimated on 2·5 g. of fat and not on 5 g. as prescribed in the standard method. Preliminary tests showed that the use of this smaller weight caused the Reichert Meissl numbers recorded in Fig. 5 to be about 2 units higher than they would have been by the standard method, but this did not in any way affect the value of the results for the purpose of detecting changes in the composition of the fat.

A study of Fig. 5 will show that for Cows 1, 2 and 3 the iodine and Reichert Meissl values appear to have been abnormal even in the control periods. Thus the iodine values, ranging from about 44 to 54, are unusually high, while the Reichert Meissl values, varying between 18 and 26 are abnormally low. Owing to the fact

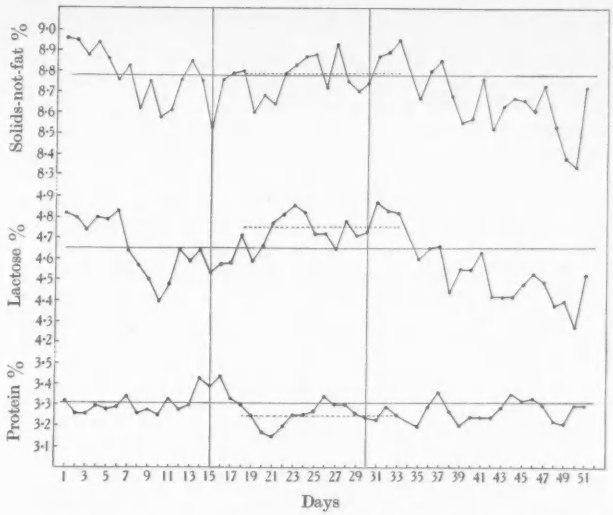

Fig. 4. The concentration of various milk constituents for Cow 4. (The straight continuous line shows the average for the pre-thyroxine period. The straight broken line shows the average from the 3rd day after thyroxine treatment began until 3 days after it ceased.)

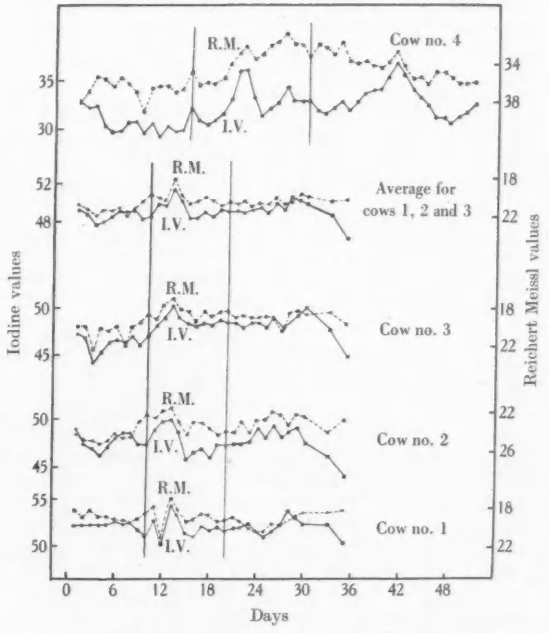

Fig. 5. The Reichert Meissl and iodine values of the milk fat.

that it was only possible to determine these values some days after the fat samples had been prepared, their apparent abnormality was not observed until the experiment with these 3 animals had been some time in progress, but, in choosing Cow 4, preliminary tests were made to ensure that for the fat which this animal secreted, these two values were well within the limits usually regarded as normal.

A detailed analysis of the apparently abnormal fat. Owing to the peculiarity of the values for the fat from the first 3 cows, it was decided to study in more detail two samples of the fat from Cow 3, one taken at the peak of total daily fat production towards the end of the thyroxine treatment (see Fig. 6), and the other taken later when the yield of fat had returned to normal. From the results recorded in Table 2 it will be observed that the low Reichert Meissl values already determined were now confirmed by the low Krischner and saponification values.

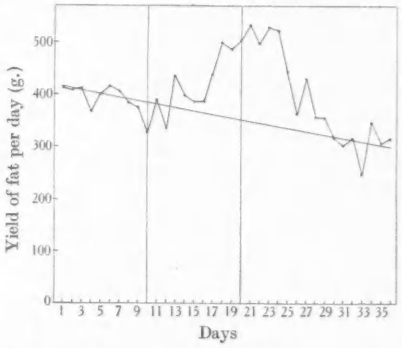

Fig. 6. The total amount of fat secreted each day by Cow 3.

Table 2. *Analytical data for pooled samples of butterfat secreted by Cow 3 during and after thyroxine treatment*

Description of sample	Reichert Meissl value	Polenske value	Krischner value	Iodine value	Saponification value
Obtained on the 9th and 10th days of thyroxine treatment; yield of fat much enhanced	17·2	1·0	14·6	48·4	213·4
Obtained on the 9th, 14th and 15th days after thyroxine treatment; yield of fat returned to normal	16·8	1·0	14·1	46·5	213·8
Typical figures for pooled milk	30	2	22	32	230

Since little is known of the variations which can occur in fats from different individual animals, such abnormal values suggested that a complete analysis of one of the samples from Cow 3 should be made in order to find how its composition varied from that of a sample having values which were somewhat closer to the average. It was therefore decided to analyse, by the ester distillation method, about 300 g. of the fat secreted some days after thyroxine treatment had ceased. For this purpose, the procedure outlined by Smith & Dastur [1938] was followed. The results are compared in Table 3 with those previously obtained by the same method for a more "normal" sample of butter fat.

Table 3. *The composition of a pooled sample of fat secreted by Cow 3 during the second control period compared with that of a "normal" fat previously analysed by the same method*

	Fat from Cow 3	A sample previously analysed
Reichert Meissl value ...	16·8	28·0
Iodine value ...	46·5	36·6
	%	%
Saturated acids:		
C_4	2·5	3·5
C_6	0·5	0·6
C_8	0·5	1·0
C_{10}	1·1	1·8
C_{12}	1·5	2·5
C_{14}	9·5	11·9
C_{16}	25·3	23·5
C_{18}	8·1	11·6
C_{20}	0·6	1·1
Total	49·6	57·5
Unsaturated acids:		
C_{10}	0·1	0·2
C_{12}	0·2	0·2
C_{14}	0·8	0·9
C_{16}	2·9	3·2
Oleic	41·5	35·9
Linoleic	2·9	1·2
C_{20}	2·1	0·8
Total	50·5	42·4
Total of all acids up to C_{14}	16·7	22·6

It will be seen from Table 3 that the low Reichert Meissl value for the present sample of fat was readily accounted for by slightly smaller concentrations of all lower components including butyric acid, whereas the high iodine value was evidently due mainly to greater proportions of oleic, linoleic and C_{20} unsaturated acids. So far, no explanation, such as stage of lactation or season of the year, has been found to account for the fact that 3 cows chosen from the herd at random should all exhibit this peculiarity, but since the general results of thyroxine administration for Cow 4 were so similar in every way to those for Cows 1, 2 and 3, it is most improbable that this slight abnormality in the fat of the first 3 animals caused erroneous conclusions to be drawn from the results.

Blood analysis

For the blood analysis, samples were taken from Cows 1, 2 and 4 at suitable intervals throughout the control and experimental periods. About 200 ml. of blood were drawn from one of the subcutaneous mammary veins into a bottle containing 0·2 g. potassium oxalate. The sample was shaken and then centrifuged for 45 min. at 2000 r.p.m. and the plasma separated from the corpuscles. Sugar was estimated in samples of the plasma by a macro form of the Hagedorn-Jensen procedure. That the administration of the hormone was having its characteristic effect on the blood sugar level is shown from the results set out in Table 4 where increases of 10, 15 and 26% in sugar level are recorded for the experimental period.

The extraction and analysis of the lipoids of the corpuscles and plasma were then carried out by the semi-macro method described by Smith [1938]. Though

more laborious to carry out, this process yields much larger amounts of the various lipoid constituents than are obtainable by any of the micro methods, and these larger amounts not only enable the mean molecular weights and iodine values of the fatty acids to be determined, but also allow a more reliable estimation to be made of the small triglyceride fraction of the plasma. It was therefore decided, as in the earlier experiments, that it would be better to analyse a few samples by this somewhat laborious process and obtain detailed and reliable results rather than to apply the more rapid micro methods to large numbers of samples and thus collect results of somewhat less value.

Table 4. *The glucose content of the blood plasma (mg./100 ml.)*

Days of thyroxine for Cows 1 and 2	Cow 1	Cow 2	Cow 4	Days of thyroxine treatment for Cow 4
Before 9	75	65	59	11
7	68	63	52	8
5	70	66	56	6
3	66	68	59	4
Average (A)	70	65	57	
During 4	75	75	64	4
6	75	76	75	8
8	76	73	76	11
10	81	75	72	14
Average (B)	77	75	72	
After 7	67	67	71	8
9	67	63	69	14
	—	—	64	17
Average	67	65	68	
% increase $(B-A)\times 100/A$	10·0	15·4	26·3	

Before beginning the present series of experiments, the accuracy of this semi-macro method, as far as total fatty acids were concerned, was compared with that of the micro process described by Stoddart & Drury [1929]. In their method the small amounts of fatty acids isolated are titrated with standard alkali and the results expressed in milliequivalents. The actual weight of fatty acids can only be obtained in this process if their mean molecular weight is known. 2212 ml. of an alcohol-ether extract of corpuscles were shown by the semi-macro method to contain 360 mg. fatty acids per 100 ml. and the mean molecular weight of the acids was found to be 313. This is equivalent to 11·5 milliequivalents of fatty acids per litre of corpuscles. Mr M. C. Manifold of the Biochemical Department, the University of Liverpool, kindly undertook to analyse independently 20-ml. samples of the same extract by the Stoddart & Drury technique. He found a value of 11·3 milliequivalents per litre, a difference of less than 1·8 %. It would appear therefore that the semi-macro process, which yields much greater amounts of the material to be studied and gives the actual weights of fatty acids present, is no less accurate than the more rapid micro methods from which fewer conclusions can safely be drawn.

In the present experiments, the alcohol-ether extracts of the plasma (1 in 20) and of the corpuscles (1 in 25) were prepared and analysed separately. A somewhat condensed form of the results appears in Tables 5 and 6. The figures in the column headed "Recovery %" in Table 5 show to what extent the total non-phosphatide lipoids were accounted for by the sum of the fatty acids, unsaponi-

fiable matter and glycerol. Most of the figures were over 92 %, but it would be difficult to state what proportion of the remainder was water-soluble fatty acids. Individual values for the various constituents were determined for Cow 2 as for Cow 1, but in order to curtail Table 5 only the means are given for this animal.

Table 5. *Analysis of the non-phosphatide lipoids of the blood plasma (mg./100 ml.)*

Period of thyroxine treatment	Total acetone-soluble lipoids	Cholesterol		Total fatty acids			Tri-glyceride fatty acids	Re-covery %
		Free	Ester	Wt.	I.V.	Mol. wt.		
Cow 1								
Before 9 days	451	48	173	158	140	294	26	96
7	415	45	168	156	139	292	29	95
5	415	48	167	151	138	294	24	97
3	404	40	166	151	139	292	26	97
Mean (A)	421	45	168	154	139	293	26	96
During 4 days	378	36	151	138	140	294	23	93
6	352	36	146	131	149	288	22	94
8	373	38	145	126	149	291	17	95
10	351	35	142	131	150	290	25	94
Mean (B)	363	36	146	131	147	291	22	94
After 7 days	385	34	156	154	140	290	37	92
9	389	34	168	150	144	288	25	95
Mean	387	34	162	152	142	289	31	94
Decrease % $(A-B)\times 100/A$	14	20	13	15	—	—	(16)	—
Cow 2								
Mean before	327	30	133	125	144	294	24	92
Mean during	290	29	118	110	147	292	21	92
Mean after	289	28	118	112	145	293	22	93
Decrease %	11	3	11	12	—	—	(12)	—
Cow 4								
Before 11 days	285	25	100	117			41	95
8	273	24	100	112			36	94
6	275	24	99	114			38	92
4	266	24	103	109			30	93
Mean	275	24	100	113	125	295	36	94
During 4 days	253	21	103	109			31	98
8	233	26	79	91			31	91
11	242	25	80	99			38	91
14	235	24	81	102			40	94
Mean	241	24	86	100	127	295	35	93
After 8 days	256	25	91	105			35	93
14	250	23	86	105			38	94
17	247	23	86	105			38	93
Mean	251	24	88	105	124	299	37	93
Decrease %	12	—	16	12	—	—	(3)	—

Discussion

The milk yields. In order to estimate the effect of thyroxine on the milk yields it is necessary to compute what the approximate yield would have been had no hormone been given. Such values have been arrived at from Figs. 1 and 2 by drawing a straight line through the points representing the yields before and after thyroxine treatment. It will be observed that the slope of the lines so drawn for the experimental cows is similar to that of the lines for the controls, and that a marked enhancement of yield took place 3 or 4 days after the injections had

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Table 6. *The phosphatides of the blood plasma and the lipoid phosphorus and cholesterol of the blood corpuscles (mg./100 ml.)*

	Plasma					Corpuscles	
Period of thyroxine treatment	Alcohol-ether-soluble phosphorus	Total matter in the phosphatide fraction	Total fatty acids: Wt.	I.V.	Mol. wt.	Alcohol-ether soluble phosphorus	Total cholesterol
			Cow 1				
Before (A)	8·9	286	136	83	324	16·0	169
During (B)	7·7	230	109	82	321	16·0	159
After	8·5	254	123	82	321	16·1	171
Decrease % $(A-B)\times 100/A$	13·0	20	20	—	—	None	6
			Cow 2				
Before	7·4	248	105	81	315	16·4	165
During	6·5	223	89	79	321	16·4	169
After	6·7	246	92	83	335	16·6	164
Decrease %	12·0	10	15	—	—	None	None
			Cow 4				
Before	5·5	212	—	79	309	14·5	154
During	4·7	187	—	77	310	14·8	139
After	4·7	190	—	79	316	15·1	151
Decrease %	15·0	12	—	—	—	None	10

begun and lasted for a similar period after they had ceased. Towards the end of the thyroxine period, the yields for Cows 1, 2, 3 and 4 were 8·9, 15·3, 14·3 and 13·6 kg. per day respectively, whereas the approximate yields which would normally have been expected were 6·1, 10·2 and 10·6 kg. The administration of thyroxine therefore resulted in increases in yield of between 28 and 50 %.

The amount of milk fat secreted. From a general inspection of the diagrams for the fat percentages shown in Figs. 2 and 4, it will be seen that although the milk yield was markedly increased during the thyroxine treatment, the level of the fat in the milk did not decrease but was well maintained near the average pre-thyroxine figure for Cow 4 and slightly above the corresponding figure for Cows 1, 2 and 3. After hormone administration had ceased, return of the milk yield to normal was accompanied by a still further rise in fat percentages for the first 3 cows, but this did not occur with the fourth animal. Nevertheless with all 4 cows the total daily yield of fat was much increased for a period of several days. The most marked effect was obtained with Cow 3, for which the results are shown in Fig. 6. In this particular instance there was not only an increase of over 50 % in the daily output of fat above that which would have been expected under normal conditions by the 21st day of the experiment, but also the yield of fat on that day was some 28 % above the highest figure recorded in the pre-thyroxine period. One of the objects of the present work was to obtain pronounced effects such as this in order that any changes taking place at the same time in the nature of the milk fat and in the level and nature of the blood lipoids might be studied.

The results so far discussed accord well with the findings of Folley & White [1936] and others whose work has already been cited.

The nature of the milk fat. From the data recorded in Fig. 5 three main conclusions may be drawn. Firstly, there is an obvious tendency for a change in the iodine value of the fat to be accompanied by an inverse change in the Reichert Meissl value. This general inverse relationship has been brought out clearly in Fig. 5 by plotting the Reichert Meissl units so that they decrease as

the ordinate is ascended, the two curves for each cow thus showing a fair degree of parallelism. A similar relationship between these two values was also very pronounced in the fat secreted by cows during inanition and described previously by Smith & Dastur [1938].

Secondly, it will be observed that for all 4 cows the two values showed a small but distinct change very soon after the beginning of the thyroxine treatment, but in the main this change was transitory. Thus the mean iodine value for the fat from the first 3 animals in the first control period was 48·7 and rose to 51·5 on the 3rd day of the hormone treatment, returning to 48·5 two days later. The corresponding Reichert Meissl value decreased from 21·2 to 18·3 and rose again to 20·7. Similarly the iodine value for the fat from Cow 4 rose from a pre-thyroxine value of 30·7 to 36·4 by the 7th day of the injections and then returned to 31·6 two days later. For this particular animal the Reichert Meissl value showed a corresponding drop from 36·6 to 32·2 by the 7th day, but instead of increasing again it tended to remain near the lower figure even for some time after the hormone treatment had ceased.

Thirdly, the results in Fig. 5 show that towards the end of the hormone treatment, i.e. on the days when the greatest enhancement of yield occurred, the nature of the fat appeared to be very similar to that secreted in the pre-thyroxine periods. Even with Cow 3, for which the yield of fat was so much enhanced (Fig. 6), the Reichert Meissl and iodine values of 19 and 48 respectively on the day of greatest yield were very little different from the pre-thyroxine values of 21 and 46. For Cow 4, the corresponding values were 32 and 33 on the day of maximum yield compared with averages of 37 and 31 for the first control period.

It is evident therefore that the increase in fat secretion brought about by thyroxine is not accompanied by anything more than exceedingly slight changes in the nature of the milk fat. And it is worth observing that this is true whether the characteristic milk fat of the animal is relatively unsaturated, as with the first 3 cows, or is much nearer the average degree of unsaturation, as with Cow 4. Nor does it appear to be true only for one particular age or stage of lactation. In fact it was only at the beginning of the treatment when the gland was becoming accustomed to the enhanced yield that any significant alterations took place, the fat evidently tending at that time to become temporarily richer in unsaturated acids and very slightly poorer in lower components. Even then, however, the changes were exceedingly small.

The concentration of non-fatty solids in the milk. For the level of non-fatty solids in the fat-free milk, Folley & White [1936] obtained an increase of about 4% during the period when the yield was enhanced owing to the injection of thyroxine. In the experiments of Jack & Bechdel [1935] and of Herman *et al.* [1938], however, this effect was not so clearly marked. In the present experiments it was therefore thought worth while to estimate the protein, lactose and total non-fatty solids in the milk in order to collect further information on this important aspect of the subject. The results expressed in terms of whole milk are shown diagrammatically in Figs. 3 and 4, and, as percentages of the fat-free milk, they are summarized in Table 7. It will be observed from Table 7 that changes in the level of total non-fatty solids were exceedingly slight. For Cows 1, 2 and 3 there appeared to be a small decrease of 0·5 to 2·1% and for Cow 4 a slight increase of 0·9%, but only with Cow 3 could the change be regarded as significant. For the lactose level there was an increase with Cows 1, 2 and 4, but practically no change with Cow 3. Again only for the one cow (no. 4) was the change significant. It was in the protein level that the tendency to alter was most consistent, statistically significant decreases of 4·1 to 6·9% being observed for the first

3 cows and a smaller change of 1% for Cow 4. Since the greatest increase in lactose content and the smallest decrease in protein level were observed for Cow 4, the animal which was nearest the beginning of its lactation, it may well be that the stage of lactation is one of the main factors which determine whether thyroxine effects a change in the level of the different non-fatty solids of the milk and whether that change is positive or negative.

Table 7. *The content of non-fatty solids in the fat-free milk before, during and after thyroxine treatment*

		Total solids-not-fat				Lactose				Protein			
	Cow no. ...	1	2	3	4	1	2	3	4	1	2	3	4
Before:	Mean (A)	9·12	8·80	9·06	9·09	4·35	4·72	4·14	4·82	3·87	3·33	4·02	3·42
	Standard deviation	0·17	0·18	0·14	0·14	0·14	0·17	0·10	0·15	0·05	0·06	0·09	0·05
During:	Mean (B)	9·05	8·78	8·93	9·09	4·34	4·71	4·09	4·91	3·79	3·23	3·82	3·37
	Standard deviation	0·11	0·14	0·13	0·11	0·13	0·08	0·07	0·09	0·06	0·05	0·08	0·05
After:	Mean (C)	9·24	8·85	9·18	8·93	4·19	4·62	4·07	4·66	4·10	3·41	4·18	3·39
	Standard deviation	0·14	0·14	0·13	0·15	0·17	0·14	0·21	0·11	0·11	0·11	0·13	0·06
The mean of A and C (D)		9·18	8·925	9·12	9·01	4·27	4·67	4·105	4·74	3·985	3·37	4·10	3·405
$\frac{B-D}{D} \times 100$		−1·5	−0·5	−2·1	+0·9	+1·6	+0·9	−0·4	+3·6	−4·9	−4·2	−6·8	−1·0

The lipoids of the blood plasma. The results recorded in Table 5 show that during the period of induced hyperthyroidism there was a general decrease in the total acetone-soluble lipoids of 14, 11 and 12% for Cows 1, 2 and 4. That both the main individual constituents contributed to this general fall is shown by the fact that ester cholesterol was reduced by 13, 11 and 16% and the total fatty acids by 15, 12 and 12%. Free cholesterol, which is in any event very small and therefore difficult to estimate accurately, appeared to decrease by 20 and 3% for Cows 1 and 2 but to remain almost constant throughout the whole experiment for Cow 4.

In lactation studies, the triglyceride fatty acids are the most important lipoid constituents of the plasma since they are now known to be the main precursor of milk fat. It will be seen from Table 5 that they also appeared to decrease by 16, 12 and 3% for the 3 cows. A detailed consideration of the results and of the method of determining them, however, will show that it would be unwise to regard these decreases as possessing the same degree of accuracy as is believed to exist in the values found for the total fatty acids and total cholesterol. The results for the triglyceride fatty acids were obtained by calculation from 4 separate estimations, the total and free cholesterol, the total fatty acids and the mean molecular weight of the fatty acids. It follows that any small unavoidable errors in the determination of these 4 sets of figures, particularly of the free cholesterol, may have combined to cause greater errors in the small values calculated for the triglyceride acids. It is not possible therefore to lay much stress on the fact that for Cow 1, for example, these particular constituents appeared to drop from a pre-thyroxine value of 26 mg./100 ml. to one of 22 during the experimental period. But, taken as a whole, it may be concluded that there certainly was no tendency for the level of the triglyceride fatty acids to rise, and that if any change at all took place, it was in the direction of a slight decrease.

The criticism may be advanced here that these values for the lipoid levels have all been determined on the venous blood coming from the mammary gland rather than on the arterial blood supplying it. Owing to the experimental difficulties involved in obtaining arterial blood it was necessary to use venous samples in the

present experiments. Actually it is unlikely that this should make any difference to the general conclusions, because recent work from various research schools, such as that by Graham *et al.* [1936], has shown that the only lipoid constituent removed from the blood as it passes through the mammary gland is a part of the triglyceride fatty acids. The average amount taken up is usually of the order of 4 to 7 mg. per 100 ml., so that, even if the rate of blood flow remained unchanged during the thyroxine treatment (and actually it usually increases), it is unlikely that anything more than some further 3 mg. of these acids would have to be removed to supply the precursors required for this enhanced yield. It is therefore probable that there would tend to be much the same relation between the values determined for the various individual lipoid levels of the blood before, during and after thyroxine administration whether the samples taken throughout were arterial or venous.

As far as the actual nature of the plasma lipoids is concerned, it is clear from the general reduction in all the main constituents that the distribution of the various lipoids was not materially altered by the hormone treatment. And from the iodine values and mean molecular weights recorded in Table 5 it is evident that there was little change in the nature of the non-phosphatide fatty acids. The slight increase in iodine value during the hormone treatment might suggest that the fatty acids tended at that time to be somewhat less saturated, but the difference must have been very small indeed. For the phosphatide fatty acids (Table 6), the iodine values and molecular weights showed too much variation among themselves for definite conclusions to be drawn. In fact here, as in earlier work [Smith, 1938], considerable difficulty was experienced in estimating the molecular weights of the phosphatide fatty acids, so that the variations shown for these particular acids are thought to be due mainly to the experimental difficulties involved in their estimation rather than to changes in the acids themselves. As is generally found for blood lipoids, the phosphatide fatty acids had higher molecular weights and much lower iodine values than those from the non-phosphatide fractions.

The corpuscle lipoids. In a previous communication by Smith [1938] decreases of considerably less than 10% were observed in the corpuscle lipoid level of lactating cows during a 12-day inanition period, as compared with a reduction of 40–50% for that of the plasma. It seemed therefore to be highly improbable that alterations could readily take place in the corpuscle lipoids even under such drastic conditions as those of a 12-day fast. Similarly, in the present work the results recorded in Table 6 show that there was evidently no detectable change in the phosphatides of the corpuscles as measured by the alcohol-ether-soluble phosphorus. The figures for total cholesterol, on the other hand, were not so uniform. There was no change for Cow 2, but with Cows 1 and 4 there appeared to be decreases of 6 and 10%. If therefore there was any alteration in the lipoid level of the corpuscles it could not have been more than a slight decrease in the cholesterol fraction, and only in Cow 4 was this really significant.

There are therefore three respects in which the results of the previous inanition and the present thyroxine experiments are similar; the corpuscle lipoids on the whole showed little change, the plasma lipoid level was markedly decreased and the general nature of the lipoids remained practically unchanged.

The possibility of a relationship existing between blood lipoid level on the one hand and the amount of milk fat secreted on the other is worthy of particular attention, since suggestions have occasionally been made that the milk fat-producing capacity of a cow might ultimately come to be judged from the concentration of fatty substances in its blood, and that even selection of calves for future

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inclusion in dairy herds might be made partly on the basis of their blood lipoid level [cf. Schoorl, 1935]. However valid such suggestions may prove, and at present confirmation is lacking, it is clear from the results just recorded that there are certain endocrinological factors which are of much greater importance than the lipoid level *per se*. Thus in the present experiments an enhanced capacity to secrete milk fat was associated not only with hyperthyroidism but also with a general decrease in the lipoid concentration in the blood. It follows therefore that little reliance can be placed on the level of total plasma lipoids in itself as a guide to the animal's capacity to produce milk fat. From the fact that the blood sugar rises during thyroxine treatment, it might have been thought that the increase in the yield of milk of almost unchanged chemical composition brought about by the hormone might be due to increases in the level of the various milk precursors in the blood. But the present results strongly suggest that, as far as fat is concerned, the enhanced yields are not dependent on increases in the level of triglycerides in the plasma. Possibly the two main factors concerned are the increased rate of blood flow and the general rise in metabolic rate which this hormone is known to cause.

At the same time it is of interest to observe that for Cow 4, which was giving the highest daily yield of fat, the triglyceride fatty acids of the plasma in the pre-thyroxine period amounted to 36 mg. per 100 ml. as compared with 26 and 24 for the other 2 animals, although the total non-phosphatide fraction was actually less for Cow 4 than for the other 2 cows. For the higher yielder, therefore, the non-phosphatide lipoids of the plasma were lower than for the other 2 animals, but the triglycerides were higher.

The fact that there was no appreciable change in the composition of the milk fat during hormone treatment in the present work suggests that the same precursors in the blood were readily available to the gland in much the same proportions throughout all the periods of the experiments, except perhaps during the first few days of the thyroxine period when the marked increase in the milk yield was actually taking place. This fact is not necessarily inconsistent with a decrease in the lipoid constituents of the plasma, for, in spite of such a decrease, the gland might still be supplied with the same amount of the same precursors, in any given time, as a result of the enhanced rate of blood flow.

There are so many factors involved in the physiological processes which lead to the secretion of milk fat that it is impossible at present to decide whether routine administration of thyroxine or breeding for animals of high thyroid activity would ever be likely in the course of time to have desirable results, but it may be concluded from the present work, taken in conjunction with the previous experiments of others, that if such a procedure did become practicable, no alteration of any importance in the nature of the butter fat would be expected, and the decreases in the plasma lipoids would not be likely to have any deleterious effects on the yield of milk or on the concentration of its constituents.

Summary

1. Thyroxine has been administered to 3 cows in the 6–9th months of their lactation under autumn conditions and to a fourth cow in its 4th month of lactation during the summer. The blood lipoids and milk fat have been studied before, during and after the hormone treatment.

2. In confirmation of the results of others it was found that the yields of milk and of milk fat were very markedly increased. There was, however, no

consistent change in the level of the non-fatty solids in the milk except for a slight decrease in the protein concentration.

3. There was no alteration of any importance in the actual nature of the milk fat during the period when its yield was enhanced except for a slight temporary change at the beginning of the hyperthyroid period, when the gland was becoming accustomed to its increased production of fat.

4. The concentration of sugar in the plasma was increased by some 10–26 % during the period of the hormone administration and this was accompanied by a general decrease of 10–20 % in the concentration of the main lipoid constituents.

5. The relationship between the lipoids of the plasma on the one hand and the yield of milk fat on the other is briefly discussed.

The authors are very much indebted to Prof. H. J. Channon and Dr N. C. Wright for the helpful interest they have taken in the work. They also wish to thank Dr A. B. Fowler and Dr P. S. Watts for assistance with the animals.

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133. ZYMOHEXASE

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With an Addendum by E. C. Bate-Smith

(*Received 29 May 1940*)

Meyerhof & Lohmann [1934, 1, 2] discovered zymohexase, an enzyme of wide distribution in animal and plant tissues which catalyses the scission of 1:6-diphosphofructose into two molecules of triosephosphate. Although they made a careful and thorough study of the properties of the enzyme and the reversibility of the reaction catalysed they made no attempt to obtain the enzyme in a purified state.

We have isolated zymohexase from rabbit skeletal muscle *ca.* 150 times more active per mg. dry weight than the original muscle. At the highest purity level 1 mg. of the enzyme catalyses the formation of 915 mg. triosephosphate per hour at 38°. The enzyme is a colourless protein accounting for some 4% of the total water-soluble protein of rabbit skeletal muscle. As yet we have no clue as to the nature of its prosthetic group.

I. *Estimation of enzyme activity*

Hexosediphosphate is converted in presence of the enzyme into two molecules of triosephosphate:

$$\text{hexosediphosphate} \rightleftharpoons 2 \text{ triosephosphate}.$$

The reaction is reversible with the equilibrium point in favour of hexosediphosphate formation under physiological conditions. It follows that the rate of triosephosphate formation cannot be used as a strict measure of enzyme concentration, since the rate will fall off as the equilibrium point is approached. To obviate this difficulty triosephosphate can be "fixed" with cyanide and the reaction then proceeds quantitatively from left to right.

Triosephosphate cyanohydrin can be estimated as inorganic phosphate after exposure to *N* soda for 20 min. at room temperature [Meyerhof & Lohmann, 1934, 1, 2]. Hexosediphosphate is not estimated under these conditions.

We have arbitrarily defined as the unit of zymohexase activity an amount which catalyses the formation of 1 mg. P equivalent of triosephosphate per 3 min. at 38° and *p*H 7·3. The test mixture was as follows: 1 ml. enzyme, 1 ml. *M*/4 HCN (neutralized to *p*H 7·3) and 1 ml. *M*/5 borate buffer *p*H 7·3. After the mixture had been equilibrated in a water bath maintained at 38°, 1 ml. *M*/20 hexosediphosphate (previously warmed to the same temperature) was rapidly introduced. At the end of 3 min. the reaction was stopped by rapid addition of 5 ml. 5% trichloroacetic acid. The inorganic P formed after alkaline hydrolysis was estimated by the method of Fiske & Subbarow [1925]. The quantity of enzyme used in the test should be between the limits of 0·5 and 1·5 units. It is important to prepare the neutralized cyanide solution fresh for each experiment.

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The sample of hexosediphosphate used in our experiments was isolated from fermenting dried yeast in the form of the acid barium salt following an unpublished method communicated to us by Prof. Robison. The barium salt was dissolved in dilute HCl and decomposed with slightly more than the theoretical amount of Na_2SO_4. The $BaSO_4$ precipitate was washed several times with water and the combined solutions were neutralized to *p*H 7·3. The final strength of the hexosediphosphate solution was determined by estimation of inorganic P after enzymic conversion into triosephosphate followed by alkaline hydrolysis.

We have used the ratio $\frac{\log I_0/I \text{ at } 280 \text{ m}\mu}{\text{enzyme units/ml.}}$ as a measure of the purity level. The density measurements were made in a 2 cm. cell. The factor for converting density values in terms of dry weight per mg. is remarkably constant throughout the entire purification process, and for practical purposes a solution containing 1 mg. dry weight of protein per ml. can be assumed to have a density of 1·8 in a 2 cm. cell.

II. *Method of isolation*

The skeletal muscle of one large rabbit (*ca.* 4 kg.) provides sufficient starting material for attaining the highest purity level. The following are the details of a typical preparation.

(1) 827 g. of fresh rabbit skeletal muscle were finely minced and mixed with 1240 ml. water. After 30 min. the suspension was filtered through muslin. The extract (1192 ml.) was treated with 357 g. $(NH_4)_2SO_4$, i.e. 30 g. for each 100 ml. The precipitate was centrifuged off and discarded. The supernatant fluid (1310 ml.) was further treated with 131 g. $(NH_4)_2SO_4$, i.e. 10 g. for each 100 ml. The precipitate was centrifuged hard for *ca.* 1 hr. and dissolved in 3·1 vol. water (final volume 500 ml.). The enzyme solution was then heated to 58° in a water bath at 70° and maintained at 58° for 2 min. The heating was so arranged that the maximum temperature was reached in <1 min. Vigorous shaking was necessary to prevent local overheating. After 2 min. exposure the solution was rapidly cooled to room temperature (time *ca.* ½ min.). The protein coagulum was filtered off with suction. The filtrate (486 ml.) was pale red though water clear. The yields of enzyme obtained in these procedures were as follows:

Stage	Volume ml.	Total units	$\frac{\log I_0/I \text{ 280 m}\mu}{\text{Enzyme units/ml.}}$
First extract	1192	15,620	5·6
After addition of 30 g. $(NH_4)_2SO_4$ per 100 ml.	1315	14,990	—
Precipitate of 2nd $(NH_4)_2SO_4$ addition	500	12,500	2·3
After heating to 58° for 2 min.	486	12,200	1·3

(2) The enzyme solution was then submitted to the first fractionation with ammoniacal $(NH_4)_2SO_4$ (6 ml. of 0·88 NH_3 to 94 ml. saturated $(NH_4)_2SO_4$). Four fractions were obtained by addition of 0·66, 0·70, 0·83 and 1·0 final volumes respectively of the $(NH_4)_2SO_4$ reagent and the precipitates were dissolved in water:

Fraction	Volume ml.	Total units	$\frac{\log I_0/I \text{ 280 m}\mu}{\text{Enzyme units/ml.}}$
I	39	3370	0·9
II	29	2540	0·6
III	23	1630	0·9
IV	33	1130	1·6

Fraction IV was discarded whereas fractions I and II were each resolved with the $(NH_4)_2SO_4$ reagent into three successive fractions, *a*, *b* and *c*. Fraction I*a* was discarded; I*b* and I*c* were retained. Fraction III*a* was retained; III*b* and III*c* were discarded. Finally all the best fractions, viz. I*b*, I*c*, II and III*a*, were combined. The final volume after the complete first fractionation series was 100 ml. containing 6750 units at the ratio 0·58 stage.

(3) The second fractionation series also involves the use of the ammoniacal $(NH_4)_2SO_4$ reagent. The additions of the reagent were so regulated as to distribute the precipitated protein into three more or less equal fractions which we shall refer to as fractions 1, 2 and 3 respectively:

Fractions	Volume ml.	Total units	$\frac{\log I_0/I \text{ 280 m}\mu}{\text{Enzyme units/ml.}}$
1	41	3200	0·60
2	50	2660	0·45
3	13	580	0·72

Fraction 3 was discarded. Fractions 1 and 2 were each resolved into two successive fractions which we shall refer to by the subscripts *a* and *b*. Fractions 1*b* and 2*a* were combined, final volume 60 ml. containing 4220 units at the ratio 0·42 stage. The yield of enzyme at this stage was 27 %.

(4) The above solution was dialysed against running water for 12 hr. and then mixed with 1/10 volume *M*/5 citrate buffer *p*H 6. Saturated $(NH_4)_2SO_4$ solution (not ammoniacal) was added in three instalments sufficient to bring the saturation to 51·5, 54 and 62 % respectively, the corresponding fractions for which we shall refer to as A, B and C:

Fraction	Volume ml.	Total units	$\frac{\log I_0/I \text{ 280 m}\mu}{\text{Enzyme units/ml.}}$
A	25	624	0·85
B	50	1764	0·36
C	40	1008	0·33

Fraction A was discarded whereas fractions B and C were combined and refractionated again into three successive fractions A^1, B^1 and C^1. The latter two together contained 800 units at the ratio 0·28 stage—the limit we have reached by our methods. The final enzyme solution was dialysed exhaustively against water, frozen at $-10°$ and dried in high vacuum over H_2SO_4. The dried enzyme which retains enzymic activity unimpaired weighed 127 mg. The yield of enzyme at the ratio 0·28 purity level was about 5 %. The degree of concentration relative to the first extract of minced muscle was $\frac{7·2}{0·28} = 26$. Assuming that the enzyme is homogeneous at the ratio 0·28 stage it follows that zymohexase accounts for *ca.* 4 % of the dry weight of the soluble protein of rabbit skeletal muscle and for *ca.* 0·7 % of the dry weight of whole muscle.

The first three stages in the method of purification (i.e. as far as ratio 0·4) are easily reproducible and there is no need to carry out estimations of activity apart from the initial one. But in proceeding from ratio 0·4 to 0·28 the purity of each fraction must be determined. The successful prosecution of the final $(NH_4)_2SO_4$ fractionations depends on a variety of factors such as the rate of stirring, the temperature of the room, the rate of addition of the reagent, etc. It is in fact a matter for experimentation in each preparation, and the element of practice is important.

III. *Properties of the homogeneous enzyme*

Elementary composition. The enzyme powder at the ratio 0·28 stage was dried at 100° *in vacuo* and found to have the following elementary composition: C, 50·6 %; H, 7·04 %; N, 15·8 %; S, 1·25 %. There was no detectable amount of ash. Phosphorus was estimated as phosphate according to Fiske & Subbarow [1925] after incineration with H_2SO_4 and H_2O_2. No trace of P was found in 15 mg. of protein, the limit of detection being 0·01 mg. The P content could not be greater than 0·05 %. Since the molecular weight of the enzyme is *ca.* 100,000 the minimum P content corresponding to 1 atom P per molecule would be 0·03 %. It is only possible to say that there cannot be more than 1 atom of P per molecule of enzyme, if that. Total carbohydrate was estimated by the method of Pirie [1936]. We are grateful to Mr R. Markham for carrying out the analysis. In 20 mg. protein, less than 0·01 mg. carbohydrate (expressed as ribose) were found, which approaches the limit of detection of the method. In a unit of molecular weight 100,000 one molecule of ribose would correspond to a carbohydrate content of 0·14 %. The value found was less than 0·05 %. No significant amount of iodine was detectable.

The analytical data rule out the possibility of zymohexase being a metalloprotein or a combination of protein and purine nucleotides. The absence of carbohydrate and P is perhaps a little surprising in an enzyme whose substrate is a phosphorylated hexose. Thus far we have been unable to detect any substance of non-amino acid nature as a part of the molecule. This does not necessarily mean or imply that the enzyme has no special prosthetic group.

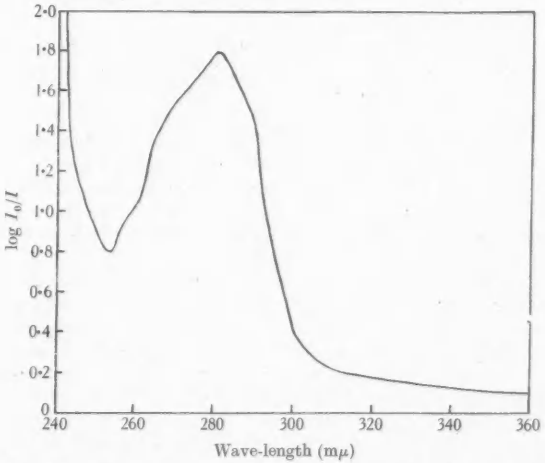

Fig. 1. Ultraviolet absorption spectrum of zymohexase concentration 0·1 %. Tube-length 2 cm.

Absorption spectrum. A solution of the enzyme showed no absorption in the visible region; however, the ultraviolet absorption spectrum was that of a typical protein (cf. Fig. 1). From the absorption spectrum in *N*/10 NaOH the tyrosine and tryptophan contents of the enzyme were calculated by the method of Holiday [1936]. There was 10·6 % tyrosine but no tryptophan.

Catalytic constants. At the extinction ratio 0·286 stage 1 mg. of the enzyme is equivalent to 6·3 catalytic units. Thus 1 mg. zymohexase under the conditions of our standard test at 38° and *p*H 7·33 forms 6·3 mg. triosephosphate-P in 3 min. or 126 mg. triosephosphate-P per hour. 1 mg. triosephosphate-P is equivalent to 5·48 mg. triosephosphate (expressed as free acid). Therefore under standard conditions, i.e. *p*H 7·3 and 38°, 1 mg. zymohexase forms 691 mg. triosephosphate per hour. At *p*H 9 and 38° the rate is 915 mg. per hour.

Assuming a molecular weight of 100,000 calculation shows that one molecule of zymohexase would form 6600 molecules of triosephosphate per min. at 38° and *p*H 7·3. At 38° and *p*H 9 the "turnover number" would be 8800.

General properties. On drying the frozen solution the enzyme is obtained in the form of a pure white feathery powder. There is no loss of activity on drying. The dried enzyme dissolves readily in distilled water, yielding a colourless, crystal clear solution. The enzyme is relatively stable either in solution or in the dried form. Aqueous neutral solutions may be kept some weeks at 0° with negligible loss of activity. The enzyme is less stable at *p*H 6 and very unstable at *p*H 4 or less. Dilute ammoniacal $(NH_4)_2SO_4$ is an especially favourable medium for preserving activity.

Temperatures higher than 50° destroy the enzyme:

Temperature	50°	60°	70°
Time of exposure (min.)	5	5	5
% inactivation	8	35	91

In presence of 10 % $(NH_4)_2SO_4$ the resistance to high temperatures is greater, e.g. heating to 58° for 3 min. in this medium produces no appreciable inactivation. This is made use of in the method of preparation.

Organic solvents must be avoided in the preparation of the enzyme. Strong aqueous solutions of alcohol and acetone inactivate the enzyme rapidly at room temperature and even at 0° or below.

Homogeneity of the purified enzyme. We have consistently failed to advance the purity of the enzyme beyond the ratio 0·28 stage. Fractionation of the enzyme at this stage merely yielded a series of identical fractions.

In the addendum, Dr E. C. Bate-Smith discusses the results obtained by cataphoresis of the enzyme in the Tiselius apparatus. At the lower stages of purity several components were observed; at the ratio 0·28 stage, there was only one component.

Mr J. St J. Philpot has examined the purified enzyme in the ultracentrifuge. Two different preparations were found to contain only a single, sharply defined component, though the sedimentation constants were not identical in the two cases. Unfortunately, the investigation could not be continued, and therefore no definite statements of molecular weight can be made on the strength of the available evidence.

The purity of our enzyme preparation was also checked by tests for other enzymes known to be present in the original muscle extract. No trace was found of the following eight enzymes; lactic, malic, α-glycerophosphoric and triosephosphoric dehydrogenases, phosphorylase, enolase, isomerase and adenylic acid deaminase. Some 10–15 mg. of the enzyme were employed in each of these tests. The triosephosphoric dehydrogenase is present in small amount at the ratio 0·6 stage; with further ammonium sulphate fractionation the impurity is easily eliminated. Thus at the ratio 0·45 stage the test for the triosephosphoric enzyme was completely negative.

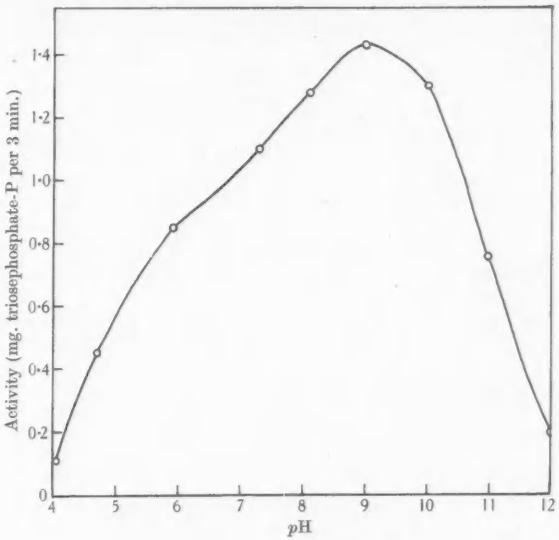

Fig. 2. *p*H-activity curve.

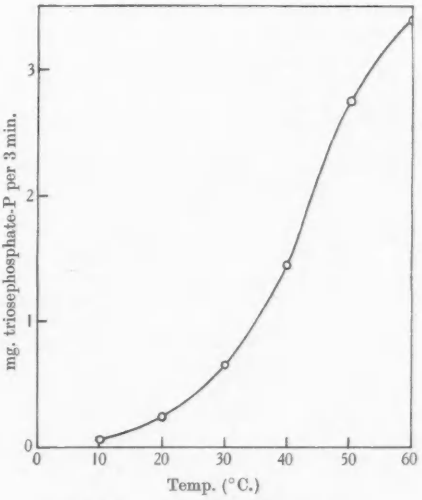

Fig. 3. Effect of temperature on velocity.

IV. *Kinetics*

Apart from the studies of Meyerhof & Lohmann [1934, 1, 2; 1935] on the reversibility of the reaction catalysed by zymohexase there is little known of the kinetics of the enzyme. This gap is in part due to the lack of a suitable method for measuring the activity of the enzyme. The cyanide fixation method described in Section I obviates the principal difficulty and has enabled us to determine the effect of various conditions on the activity of the enzyme.

The *p*H-activity curve is shown in Fig. 2. The enzyme is active over a wide range of *p*H with a maximum at *ca.* *p*H 9. The activity at *p*H 11 is *ca.* 50% of the activity at *p*H 9. Even at *p*H 12 the enzyme shows some activity. Activity falls off rapidly on the acid side of neutrality. These facts may be correlated with the marked stability of the enzyme to alkali, and its rapid denaturation at *p*H 4 and below.

Between 30° and 50° the Q_{10} is very nearly 2 (cf. Fig. 3). Below 30° the Q_{10} is greater than 2 and above 50° it is less than 2. In the higher range of temperature increased velocity is of course compensated by increased rate of destruction during the course of the experiment.

The rate of formation of triosephosphate is practically independent of the initial concentration of hexosediphosphate (cf. Table 1). The Michaelis constant, i.e. the half-speed concentration, is less than 0·001 *M*.

Table 1. *Effect of substrate concentration*

Hexosediphosphate concentration (*M*)	Velocity, in mg. P per 3 min.
0·005	1·25
0·0083	1·34
0·0166	1·36
0·033	1·43
0·05	1·47

The additions in each case were 1 ml. enzyme, 1 ml. borate buffer *p*H 7·3, 1 ml. *M*/4 KCN and varying amounts of hexosediphosphate; final volume 5 ml. Temp. 38°.

Table 2. *Effect of enzyme concentration*

Enzyme ml.	Time min.	Triosephosphate-P found (mg.)
0·2	15	0·66
0·5	6	0·92
1·0	3	1·04
2·0	1·5	1·08

The additions in each case were, 1 ml. *M*/20 hexosediphosphate, 1 ml. *M*/4 KCN, 1 ml. borate buffer *p*H 7·3, and varying amounts of enzyme; total volume 5 ml. Temp. 38°.

The effect of cyanide concentration on the velocity of triosephosphate formation has an important bearing on our standard test of enzyme activity. The final cyanide concentration must be 0·05 *M* for the reaction velocity to be maximal:

HCN concentration (*M*)	0	0·01	0·025	0·05	0·1
mg. triosephosphate-P formed in 3 min.	0·4	0·72	0·97	1·11	1·12

The velocity of triosephosphate formation is proportional to the concentration of enzyme only within restricted limits under the conditions of our standard test (cf. Fig. 4). In the range 0·5 to 1·5 enzyme units a linear relation obtains. The lack of proportionality at higher enzyme concentrations is referable

to the exhaustion of substrate before the end of the experimental period. When employing low enzyme concentrations the experimental period has to be extended in order to obtain sufficient P for accurate estimation. There is clearly destruction of the enzyme in the course of prolonged contact with the substrate (Table 2).

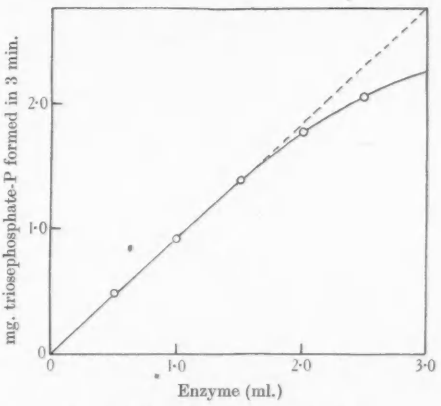

Fig. 4. Effect of enzyme concentration.

V. *Specificity of substrate*

Zymohexase does not attack glucose, fructose or fructose-6-phosphate. Solutions of glucose and fructose in presence of the enzyme show no change in optical relation over a period of 24 hr. Under the conditions of the standard test fructose-6-phosphate does not yield alkali-labile P.

Lohmann [1935] discovered in muscle extract "aldolase", an enzyme which catalyses the aldol condensation of dihydroxyacetonephosphate with various aliphatic or aromatic aldehydes, e.g.

dihydroxyacetonephosphate + *d*-glyceraldehyde → fructose-1-phosphate
dihydroxyacetonephosphate + acetaldehyde → 5-desoxyxyloketose-1-phosphate.

The analogy between the catalytic actions of zymohexase and aldolase respectively was sufficiently close as to lead the discoverer to the surmise that they were the same enzyme. This point we have been able to clinch by the demonstration that preparations of zymohexase at the highest purity level still manifest strong aldolase activity.

The method of testing for aldolase activity is a little complicated and requires a few explanatory remarks. Hexosediphosphate in presence of zymohexase is partially and reversibly resolved into one molecule each of dihydroxyacetonephosphate and glyceraldehydephosphate. In absence of a ketone fixative the reaction does not go to completion. If aldolase is present addition of acetaldehyde should result in the formation of methyltriosephosphate by combination with dihydroxyacetonephosphate. The new compound formed contains alkali-labile P and can be estimated as triosephosphate. The removal of dihydroxyacetonephosphate by aldol condensation will disturb the zymohexase equilibrium and more will be formed to satisfy the equilibrium requirements. The net result should be therefore an increase in alkali-labile P after addition of

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acetaldehyde. Table 3 summarizes a typical experiment demonstrating increased production of alkali-labile P in presence of various concentrations of acetaldehyde.

Table 3. *Aldolase reaction*

In all experiments, 2 ml. enzyme, 1 ml. buffer *p*H 7·3, 2 ml. *M*/20 hexosediphosphate. Temp. = 38°.

Exp.

1 Incubated 10 min.
2 Incubated 20 min.
3 Incubated 10 min., then 4·4 mg. acetaldehyde added, incubated a further 10 min.
4 Incubated 10 min., then 17·6 mg. acetaldehyde added, incubated a further 10 min.
5 Incubated 10 min., then 88 mg. acetaldehyde added, incubated a further 10 min.

Triosephosphate-P found (mg.)

Exp. 1. 0·50. Exp. 2. 0·50. Exp. 3. 0·74. Exp. 4. 1·15. Exp. 5. 1·94

VI. *Products of reaction*

Meyerhof & Lohmann [1935] originally formulated the catalytic scission of fructosediphosphate as follows:

fructosediphosphate $\rightleftharpoons$ 2 dihydroxyacetonephosphate.

The basis of this formulation was the observation that the triosephosphate formed in crude muscle extracts was more than 95% dihydroxyacetonephosphate. Later Meyerhof & Kiessling [1935] discovered in muscle extracts, isomerase, an enzyme which catalyses the following reactions:

d-3-glyceraldehydephosphate $\rightleftharpoons$ dihydroxyacetonephosphate.

It thus became clear that the presence only of the ketotriosephosphate in muscle extracts did not necessarily exclude the formation of the aldotriosephosphate. Since the isomerase equilibrium is vastly in favour of the former, no appreciable quantity of aldotriosephosphate should be present in a solution containing both zymohexase and isomerase.

It has now become possible to test this hypothesis directly. Purified zymohexase preparations which are free from isomerase should catalyse the formation of equal quantities of the two triosephosphates. Both the aldo- and keto-triosephosphates contain alkali-labile P. But whereas the aldotriosephosphate is oxidized by iodine to phosphoglycerate whose P is with difficulty hydrolysable, the ketotriosephosphate is unaffected by iodine. We should expect therefore that half the total triosephosphate formed from fructosediphosphate should disappear after iodine treatment. Some typical results are shown in Table 4. It is clear that within the limits of experimental error iodine treatment halves the total alkali-labile P.

Table 4. *Ratio of glyceraldehydephosphate to dihydroxyacetonephosphate*

Enzyme and hexosediphosphate were incubated for 10 min. at 37° without addition of cyanide. Aliquots were then estimated for alkali-labile P before and after oxidation with iodine.

Exp.	Glyceraldehyde-phosphate-P mg.	Dioxyacetone-phosphate-P mg.	Ratio
1	0·187	0·131	59/41
2	0·22	0·13	63/37
3	0·49	0·67	42/58
4	0·25	0·25	50/50
5	0·21	0·30	41/59
		Mean	51/49

VII. *The equilibrium constant*

The equilibrium constant K for the reactions catalysed by zymohexase is given by the equation

$$(1)\quad K=\frac{[\text{dihydroxyacetonephosphate}]\,[\text{glyceraldehydephosphate}]}{[\text{hexosediphosphate}]}.$$

Since the two triosephosphates are formed in equal quantity,

$$(2)\quad K=\frac{[\frac{1}{2}\text{ total triosephosphate}]^2}{[\text{hexosediphosphate}]}.$$

K has the dimensions of a concentration and will be expressed in terms of g. mol. per litre. As in all dissociating systems, dilution of the system at equilibrium will bring about an increased breakdown of hexosediphosphate.

Solutions of enzyme and hexosediphosphate were incubated at various temperatures until equilibrium had been reached. The total triosephosphate was then estimated as alkali-labile P. The amount of hexosediphosphate remaining was obtained by subtraction of the triosephosphate-P from the original total P. In these experiments the quantity of enzyme used was sufficient to permit the attainment of final equilibrium within 5–10 min. The instability of triosephosphate would otherwise prove an interfering factor. Table 5 shows that K is independent of the concentration of enzyme (within the limits of experimental error), and that the enzyme obeys the essential criterion of a true catalyst. K is also independent of the initial substrate concentration within the limits of concentration tested. The small variations in K are well within the limits of experimental error. The amount of triosephosphate present at equilibrium is small, and this not too easily estimated quantity must be halved and then squared in the calculation of K. Although K remains the same for all substrate-concentrations, the percentage of hexosediphosphate converted into

Table 5. *Effect of enzyme concentration on the equilibrium constant*

ml. enzyme	mg. triosephosphate-P at equilibrium	mg. hexosediphosphate-P at equilibrium	K
0·3	0·311	2·51	$1·24\times10^{-4}$
1·0	0·293	2·53	$1·21\times10^{-4}$
3·0	0·292	2·53	$1·21\times10^{-4}$

The reaction mixture contained in each case 1 ml. $M/20$ hexosediphosphate, 1 ml. $M/4$ borate buffer pH 7·33, and varying amounts of enzyme; final volume 5 ml. Temp. 38°. Values of K in g. mol./litre. All values are the means of duplicate observations.

Table 6. *Effect of substrate concentration on the equilibrium constant*

Initial hexosediphosphate concentration M	mg. triosephosphate-P at equilibrium	mg. hexosediphosphate-P at equilibrium	$\frac{\text{Triosephosphate-P}}{\text{Initial hexosediphosphate-P}}\times100$	K
0·005	0·231	1·15	16·3%	$1·30\times10^{-4}$
0·01	0·305	2·52	11·5%	$1·19\times10^{-4}$
0·02	0·435	5·18	8·3%	$1·21\times10^{-4}$
0·03	0·534	7·93	6·3%	$1·16\times10^{-4}$

All reaction mixtures contained 1 ml. enzyme, 1 ml. borate buffer pH 7·33, and varying amounts of hexosediphosphate, in a total volume of 5 ml. Temp. 38°. All values are the means of duplicate estimations, except those for 0·01 M hexosediphosphate, which are the means of 7 different estimations. K is expressed in g. mol./litre.

Table 7. *Effect of temperature on the equilibrium constant*

Temp.	mg. triose-phosphate-P at equilibrium	mg. hexose-diphosphate-P at equilibrium	$\frac{\text{Triosephosphate-P}}{\text{Initial hexosediphosphate-P}} \times 100$	K
28°	0·220	2·60	7·8	$6{\cdot}0 \times 10^{-5}$
38°	0·305	2·52	11·5	$1{\cdot}19 \times 10^{-4}$
48°	0·417	2·40	14·8	$2{\cdot}34 \times 10^{-4}$

All reaction mixtures contained 1 ml. enzyme, 1 ml. borate buffer *p*H 7·2, and 1 ml. *M*/20 hexosediphosphate, in a total volume of 5 ml. All values are the means of duplicate estimations except those for 38°, which are the means of 7 different estimations. *K* is expressed in g. mol./litre.

triosephosphate decreases with increased substrate concentration. As can be deduced from equation (3) below, the percentage of triosephosphate formed is proportional to the square root of the dilution (Fig. 5).

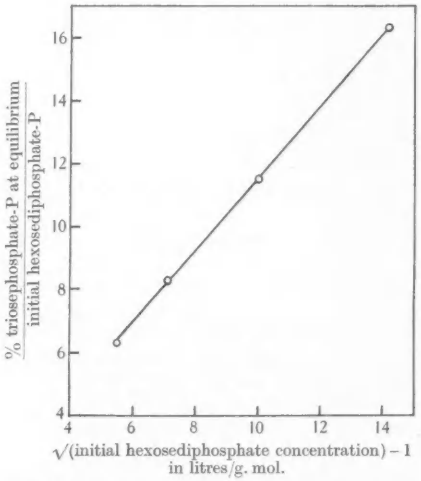

Fig. 5. Effect of hexosediphosphate concentration on equilibrium. Details as in Table 5.

The effect of temperature on the equilibrium constant is shown in Table 7. According to the van't Hoff isochore, the plot of log *K* against $1/T$ should yield a straight line. The linear relation found is shown in Fig. 6.

The heat of reaction can be calculated from the equation:

$$\ln K_1 - \ln K_2 = -\frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right).$$

The values found are:

Temp. range	ΔH in g. cal.
28°–38°	12,720
38°–48°	13,580
28°–48°	13,130

Meyerhof & Lohmann [1935], by direct calorimetric measurements on muscle extracts, found $\Delta H = 14{,}000$ g. cal. The presence of isomerase in muscle extracts

does not appear to affect the value of ΔH; the conversion of glyceraldehyde-phosphate into dihydroxyacetonephosphate must therefore involve little heat change.

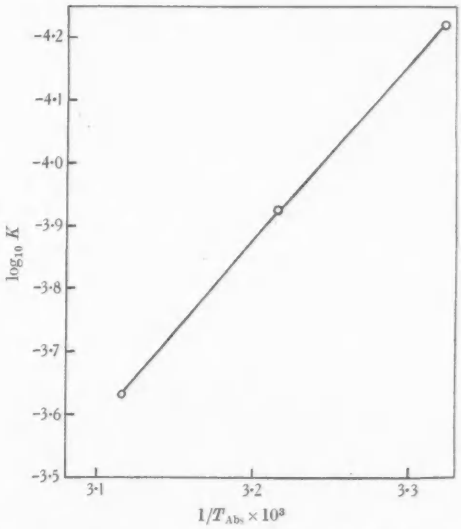

Fig. 6. Variation of equilibrium constant with temperature.

The free energy change for the conversion of hexosediphosphate into triose-phosphate can be calculated from the relation:

$$\Delta F = -RT \ln K.$$

The values for different temperatures are:

Temp.	ΔF in g.-cals.
28°	5820
38°	5590
48°	5340

VIII. *Isomerase equilibrium*

The equilibrium between dihydroxyacetonephosphate and glyceraldehyde-phosphate catalysed by isomerase has hitherto been difficult to evaluate. No technique is available for estimating either of the two triosephosphates with the required degree of accuracy. The only quantity that can be determined with reasonable accuracy is the sum of the two triosephosphates.

From a knowledge of the equilibrium constant $K_{\text{zymohexase}}$ (cf. equation (1)) it is possible to arrive at $K_{\text{isomerase}}$ by the following indirect method. Hexose-diphosphate is added to dialysed muscle extract and the equilibrium ratio $\frac{\text{total triosephosphate}}{\text{nitial hexosediphosphate}}$ is then measured.

This will be different from the ratio obtained with purified zymohexase. The presence of isomerase in the crude muscle extract is responsible for the alteration in the ratio. The calculation is made as follows.

If a = g.mol. hexosediphosphate present initially,
$a-x$ = g.mol. hexosediphosphate present at equilibrium,
$x-y$ = g.mol. glyceraldehydephosphate present at equilibrium,
$x+y$ = g.mol. dihydroxyacetonephosphate present at equilibrium,
$2x$ = g.mol. total triosephosphate at equilibrium,
V = volume.

then
$$K_{\text{zymohexase}} = \frac{(x+y)(x-y)}{(a-x)V}, \qquad \ldots(3)$$

$$K_{\text{isomerase}} = \frac{x+y}{x-y}. \qquad \ldots(4)$$

Therefore
$$y = \frac{K_{\text{isomerase}}-1}{K_{\text{isomerase}}+1}x. \qquad \ldots(5)$$

Substituting this value of y in equation (3) and simplifying,

$$K_{\text{zymohexase}} = \frac{\frac{4x^2 K_{\text{isomerase}}}{(K_{\text{isomerase}}+1)^2}}{(a-x)V}. \qquad \ldots(6).$$

Therefore
$$K_{\text{isomerase}}^2 + \left(2 - \frac{4x^2}{(a-x)VK_{\text{zymohexase}}}\right) K_{\text{isomerase}} + 1 = 0. \qquad \ldots(7)$$

To solve this quadratic equation for $K_{\text{isomerase}}$ the only quantity required is x, which is half the sum of the total triosephosphate. $K_{\text{zymohexase}}$ is known.

The following are the protocols of a typical experiment designed to evaluate $K_{\text{isomerase}}$. Dialysed muscle extract (1 ml.) was mixed with 1 ml. of 0·0091 M hexosediphosphate (2·82 mg. P) in a final volume of 5 ml. at 38° and pH 7·3. At equilibrium the triosephosphate-P formed was 0·84 mg., and the hexosediphosphate-P remaining was 1·98 mg. Therefore $x = 2{\cdot}71 \times 10^3$ mol. and

$$(a-x) = 6{\cdot}38 \times 10^3 \text{ mol.}$$

and the ratio
$$\frac{\text{total triosephosphate-P}}{\text{initial hexosediphosphate-P}} 100 = 29{\cdot}8\,\%.$$

These results are in good agreement with those of Meyerhof & Lohmann [1934, 1]. The ratio for purified zymohexase under the same experimental conditions was 11·5 %. Substituting the values for x and $a-x$ in equation (7), we find that $K_{\text{isomerase}} = 36{\cdot}3$,

i.e. the ratio
$$\frac{\text{glyceraldehydephosphate}}{\text{total triosephosphate}} 100 = 2{\cdot}7\,\%.$$

From $K_{\text{isomerase}}$ the free energy of the conversion of glyceraldehydephosphate into dihydroxyacetonephosphate can be calculated. At 38°, $\Delta F = -2220$ g. cal.

IX. *Inhibitors*

Table 8 summarizes the effects of various agents on zymohexase. $Na_2S_2O_4$, ascorbic acid, reduced and oxidized glutathione, iodoacetic acid and H_2O_2 have no appreciable effect. There is no suggestion therefore of an oxidizable or reducible group being concerned in the activity of the enzyme.

Iodine in very low concentration inhibits the enzyme completely. No correlation was observed between the degree of inhibition and the number of tyrosine molecules in the enzyme.

The inhibitory effect of heavy metals has an important practical bearing on the purification of the enzyme. There are sufficient amounts of heavy metals

Table 8. *Effect of various substances on zymohexase activity*

Reagent	Final concentration	% inhibition
Sodium hydrosulphite	0·1%	0
Ascorbic acid	0·1%	0
Reduced glutathione	0·1%	0
Iodoacetic acid	*M*/500	0
H_2O_2	*M*/500	0
I_2	*M*/50,000	100
I_2	*M*/150,000	46
I_2	*M*/500,000	18
$AgNO_3$	*M*/50,000	100
$CuSO_4$	*M*/50,000	100
$ZnSO_4$	*M*/50,000	34
$Hg(NO_3)_2$	*M*/50,000	16

In each experiment, 1 ml. enzyme was incubated with 1 ml. of the various substances for 5 min. at 38°. Buffer, cyanide and hexosediphosphate were then added, and the activity tested in the usual way. A control was run simultaneously.

in commercial $(NH_4)_2SO_4$ to cause considerable inactivation of the enzyme, especially at higher purity levels. It is essential to use analytical reagents and glass-distilled water throughout the purification process.

Glucose, fructose and fructose-6-phosphate, although not attacked by the enzyme, competitively inhibit the scission of hexosediphosphate (cf. Table 9). Fructose-6-phosphate shows this phenomenon best. α-Glycerophosphate at the same concentration has no effect on the enzyme.

Table 9. *Competitive inhibition*

Each experiment contained 1 ml. enzyme, 1 ml. borate buffer, *p*H 7·3, 1 ml. *M*/4 KCN, 1 ml. *M*/10 hexosediphosphate and 1 ml. of the various compounds listed below.

Substance added	None	*M*/10 glucose	*M*/10 fructose	*M*/10 fructose-6-phosphate	*M*/10 β-glycero-phosphate
mg. triosephosphate-P found in 3 min.	1·29	1·22	1·08	0·89	1·28
% inhibition	—	5·4	16·3	31	0

X. *Crystalline form*

In the course of the purification of zymohexase a marked tendency of the $(NH_4)_2SO_4$ precipitates to take on crystalline form was observed. After some hours the precipitates show pronounced anisotropy of flow when examined with polarized light. The crystals which form are very small needles. During the final stages of the purification of the enzyme the $(NH_4)_2SO_4$ precipitates show no tendency to form these crystals and it must be concluded therefore that some protein other than the enzyme is responsible for the anisotropic crystals.

Recently Baranowski [1939] and Bailey [1940] have isolated from rabbit skeletal muscle a protein (the so-called "myogen B") whose general properties resemble closely those of the crystalline material found in impure preparations of zymohexase. Through the kindness of Dr Bailey we had an opportunity of testing a highly purified crystalline preparation of this protein for zymohexase activity. No trace of activity was found.

It is an interesting commentary on the value of the crystalline state as an index of purity of proteins that one of the best tests for the homogeneity of zymohexase preparations is that the $(NH_4)_2SO_4$ precipitates should show no tendency to crystallize. As long as the precipitate shows anisotropy of flow

in polarized light the enzyme is still inhomogeneous. It would appear from our results that this so-called "myogen B" tends to crystallize even when present to the extent of only 10% of the total protein. At this stage the crystals of course are grossly contaminated with other proteins.

XI. *Zymohexase and muscle proteins*

There is constant reference in the literature to the number of proteins present in skeletal muscle. Myosin, myohaemoglobin, myoalbumin, myogens A and B and globulin X are assumed to represent the principal proteins of skeletal muscle. This view is somewhat naïve in view of our knowledge of the enzymic equipment of skeletal muscle. One could enumerate some fifty enzymes known to be present in skeletal muscle. Making the not unjustifiable assumption that all these enzymes are proteins it follows that at least fifty different proteins should be found in skeletal muscle. The concentration will vary from one enzyme to another. Some like zymohexase are present in relatively high concentration (*ca.* 5% of the total water-soluble protein) whereas others like catalase are present only in minute concentration. There can be no question therefore that what have hitherto been regarded as the main proteins of skeletal muscle are merely mixtures of enzymes (or other proteins) with superficially similar properties.

We are grateful to Dr Godden and Miss Simpson of the Rowett Research Institute for carrying out iodine estimations on the enzyme.

Two of us (D. H. and H. G.) are indebted to the Department of Scientific and Industrial Research for Maintenance Grants.

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ADDENDUM

CATAPHORETIC BEHAVIOUR OF ZYMOHEXASE

By E. C. Bate-Smith

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The water-soluble proteins of rabbit muscle show two distinct boundaries in the Tiselius apparatus. Two preparations made by extracting the minced muscle with 7% LiCl solution and dialysing against distilled water until no further precipitation of globulins occurred gave the following data. The cataphoresis was carried out in 0·05*M* phosphate buffer at *p*H 7·0.

	Electrophoretic mobility $\times 10^5$ cm.^{2} volt^{-1} sec.$^{-1}$)	
	Boundary 1	Boundary 2
Preparation 1	−5·3	−1·6
Preparation 2 (*a*)	−5·4	−1·6
(*b*)	−5·2	−1·6

These fractions corresponded in properties to myoalbumin and myogen, respectively [cf. Bate-Smith, 1937]. Myogen, the protein forming boundary 2, was definitely not homogeneous.

Preparations of zymohexase were submitted by Dr Green and his co-workers. Successive preparations were of increasing activity and the cataphoretic results showed them to be increasingly homogeneous. The final preparation appeared to be completely homogeneous. A fast-moving fraction (at *p*H 7) in the first preparation was absent from the later ones. The mobility of the main fraction showed that it formed part of the myogen complex. The cataphoretic data obtained with these preparations were as follows.

Preparation	Activity ratio	*p*H	Electrophoretic mobility $\times 10^5$ main boundary
1	0·6	7·4	−1·1
2	0·4	6·5	−0·6
		6·0*	+0·7
3	0·34	6·0*	+0·85
4	0·28	7·0	−0·85
		6·0*	+0·35

* Prolonged cataphoresis at *p*H 6·0 leads to inactivation of the enzyme.

Judging from preparations 2 and 4, the main constituent of the zymohexase preparation has an isoelectric point in 0·05 *M* phosphate at *p*H ~ 6·3. This was confirmed by observing the cataphoretic behaviour of particles of heat-coagulated enzyme in the apparatus described by Dummett and Bowden. The particles were isoelectric at *p*H 6·3. The isoelectric point of "myogen" has been reported by different observers to lie between 6·3 [Weber, 1925] and 6·7 [Bate-Smith, 1937]. Zymohexase is therefore one member of the complex of substances forming the myogen fraction, its isoelectric point lying at the most acid end of the range covered by the group. In bulk it probably represents about 10 % of the whole myogen complex, which again forms 10 % of the total muscle protein. It is interesting to note that Finn [1932] obtained strong evidence of the inhomogeneity of myogen now confirmed in several quarters.

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134. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

67. THE MOLECULAR CONSTITUTIONS OF CATENARIN AND ERYTHROGLAUCIN, METABOLIC PRODUCTS RESPECTIVELY OF *HELMINTHOSPORIUM CATENARIUM* DRECHSLER AND OF SPECIES IN THE *ASPERGILLUS GLAUCUS* SERIES

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AND HAROLD RAISTRICK

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(*Received 27 May 1940*)

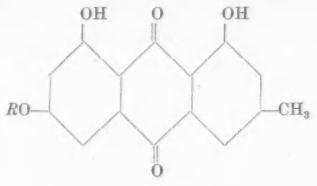
CHARLES *et al.* [1933] isolated from the mycelium of *Helminthosporium gramineum* Rabenhorst two hitherto undescribed hydroxyanthraquinones which they named helminthosporin (4:5:8-trihydroxy-2-methylanthraquinone) and hydroxy-*iso*helminthosporin, $C_{15}H_{10}O_6$. Since the latter substance was found by Raistrick *et al.* [1934] to be the main colouring matter of *Helminthosporium catenarium* Drechsler its name was altered by these workers to catenarin. Catenarin was shown by them to contain four hydroxyl groups, to give 2-methylanthracene on zinc dust distillation and, for reasons for which the original work must be consulted, was believed to be a 1:5:8-trihydroxy(hydroxymethyl)-anthraquinone with the hydroxymethyl group either in position 2, 6 or 7.

We have now shown that when catenarin is reduced with hydriodic acid and red phosphorus, emodin anthranol is formed in good yield and gives, on oxidation with chromic acid, *Frangula*-emodin, $C_{15}H_{10}O_5$, 4:5:7-trihydroxy-2-methylanthraquinone (I). Hence it follows that catenarin must be a hydroxyemodin and must therefore be II, III, IV, V or VI. Of these II is at once excluded since this must be reserved for ω-hydroxyemodin, a metabolic product of a strain of *Penicillium cyclopium* Westling [Anslow *et al.* 1940] and ω-hydroxyemodin and catenarin are quite different substances. The belief that catenarin contains a side chain —CH_3 group and not a —CH_2OH group is confirmed by the fact that it gives 1 mol. of acetic acid in a Kuhn-Roth oxidation.

The following experimental facts previously established by Raistrick *et al.* [1933; 1934] lead to the exclusion of structures III, V and VI for catenarin and support the view that catenarin is IV.

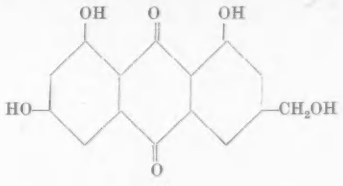
(*a*) From the similarity of the absorption curves in conc. H_2SO_4 of, on the one hand, helminthosporin and cynodontin each of which has been conclusively shown to contain nuclear hydroxyl groups in the 1:4:5-positions, and, on the other hand, catenarin, it is logical to conclude that catenarin is also a derivative of 1:4:5-trihydroxyanthraquinone. Only IV and V satisfy this condition.

(*b*) Catenarin gives a fluorescent solution in acetic acid and this fluorescence disappears on reduction to emodin. Raistrick *et al.* [1934] have shown that this



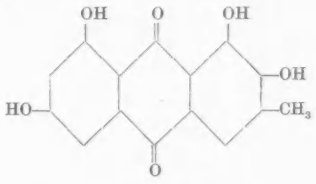
I

R=H, *Frangula*-emodin.
R=CH_3, physcion.

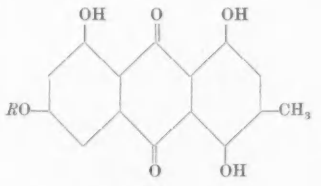


II

ω-Hydroxyemodin.

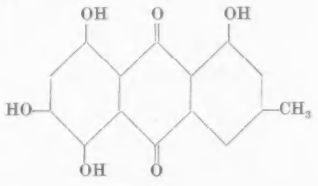


III

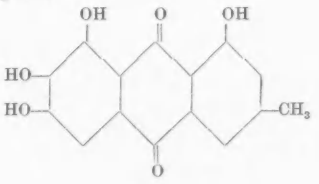


IV

R=H, catenarin.
R=CH_3, erythroglaucin.



V



VI

fluorescence, among polyhydroxyanthraquinones, is characteristic of derivatives of quinizarin, 1:4-dihydroxyanthraquinone. Only IV and V satisfy this condition.

(*c*) Catenarin is not a strong mordant dye and hence cannot be a derivative of alizarin, 1:2-dihydroxyanthraquinone. Only IV satisfies this condition.

(*d*) Charles *et al.* [1933] showed that treatment of catenarin tetra-acetate with chromic acid in acetic anhydride-acetic acid solution failed to oxidize the side chain to carboxyl and we have confirmed this observation. Several workers have attempted to carry out a similar oxidation of the acetates of polyhydroxy-methylanthraquinones with the following results.

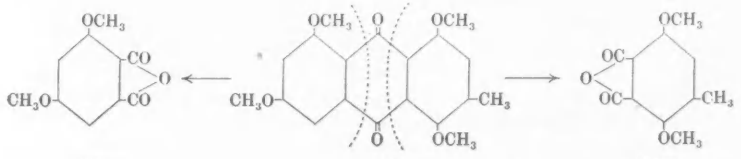
(I) *Successful oxidations.* (1) *Frangula*-emodin, 4:5:7-trihydroxy-2-methyl-anthraquinone, to emodic acid, 4:5:7-trihydroxyanthraquinone-2-carboxylic

acid [Fischer & Gross, 1911; Anslow *et al.* 1940]. (2) ω-Hydroxyemodin 4:5:7-trihydroxy-2(hydroxymethyl)-anthraquinone, to emodic acid [Anslow *et al.* 1940]. (3) Helminthosporin, 4:5:8-trihydroxy-2-methylanthraquinone to helminthosporic acid, 4:5:8-trihydroxyanthraquinone-2-carboxylic acid [Charles *et al.* 1933].

(II) *Unsuccessful oxidations.* (1) Morindone, 1:5:6-trihydroxy-2-methylanthraquinone [Simonsen, 1918]. (2) Cynodontin, 1:4:5:8-tetrahydroxy-2-methylanthraquinone [Raistrick *et al.* 1933]. The accepted explanation for these differences is that the presence of a nuclear hydroxyl group adjacent to a methyl or hydroxymethyl group prevents the oxidation taking place, e.g. morindone and cynodontin, while oxidation proceeds smoothly in compounds not containing a so-placed nuclear hydroxyl group, e.g. emodin, ω-hydroxyemodin and helminthosporin. Only III and IV contain a nuclear hydroxyl group adjacent to the methyl group.

(*e*) 1:4:5:7-Tetrahydroxy-6-methylanthraquinone has been synthesized by Charlesworth & Robinson [1934] by an unequivocal method. This substance is not identical with catenarin but it gives colour reactions which are so similar to those given by catenarin as to justify the belief that the four hydroxyl groups are in the same positions in the two compounds. Thus to quote Charlesworth & Robinson "The following are the points of divergence noted. The colour in H_2SO_4 is slightly bluer than that of catenarin. Alkaline solutions have identical colours, but those of catenarin are more rapidly oxidized in air. The orange alcoholic solution of the synthetic compound exhibits a very feeble fluorescence, that of catenarin being much more pronounced. The synthetic compound is slightly less soluble in aqueous alcohol." There are only four possible 1:4:5:7-tetrahydroxy-methylanthraquinones and of these only the one having the methyl group in position 2 (IV) could possibly give *Frangula*-emodin on removal of one hydroxyl group by reduction.

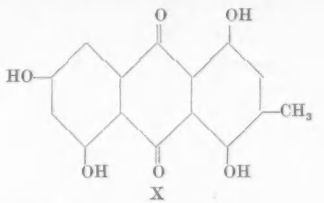
Hence it is seen that, of the four possible structures for catenarin, i.e. III, IV, V and VI, only IV satisfies all the conditions laid down in (*a*), (*b*), (*c*), (*d*) and (*e*) above. The final proof that IV is in fact the correct structural formula for catenarin was obtained as follows. Catenarin tetramethyl ether (VII) was oxidized with chromic acid in acetic acid-acetic anhydride solution and 10% of *4-methyl-3:6-dimethoxyphthalic anhydride* (VIII) and 2% of 3:5-dimethoxyphthalic anhydride (IX) were isolated from the products of oxidation.



IX VII VIII

The origin of VIII and IX from VII, by cleavage at the dotted lines, is clear and further it is obvious that, of the four possible structures III, IV, V and VI, only the tetramethyl ether of IV could possibly give rise to VIII and IX.

Finally, although it is theoretically possible to couple together the nuclei in VIII and IX to give the tetramethyl ether of a polyhydroxyanthraquinone of structure X, catenarin cannot have this structure since X could not give *Frangula*-emodin (I, $R = H$) on reduction.



X

Catenarin is therefore to be regarded as *1:4:5:7-tetrahydroxy-2-methylanthraquinone* (IV). The hydroxyl group in catenarin which is removed on reduction with HI to emodin is thus the one in position 1.

4-Methyl-3:6-dimethoxyphthalic anhydride, previously unknown, was synthesized, for comparison with the degradation product from catenarin tetramethyl ether, by a method similar to that used by Thiele & Meisenheimer [1900] and Thiele & Gunther [1906] for the synthesis of 3:6-dimethoxyphthalic anhydride from benzoquinone. Toluquinone was treated with nascent HCN in ethanol to give *2:5-dihydroxy-3:4-dicyanotoluene* which on methylation gave *2:5-dimethoxy-3:4-dicyanotoluene*. This substance, on hydrolysis with H_2SO_4, yielded directly *4-methyl-3:6-dimethoxyphthalic anhydride* (VIII).

Ashley *et al.* [1939] isolated from the mycelium of a large number of species in the *Aspergillus glaucus* series two polyhydroxyanthraquinones, i.e. physcion, 4:5-dihydroxy-7-methoxy-2-methylanthraquinone (I, $R=CH_3$) and a hitherto undescribed mould metabolic product, erythroglaucin. Erythroglaucin, $C_{16}H_{12}O_6$, was shown to be a monomethyl ether of a tetrahydroxy-methylanthraquinone, the fact that it contains a $—CH_3$ and not a $—CH_2OH$ side-chain following from a Kuhn-Roth estimation. We have now shown that *catenarin tetramethyl ether* is identical with erythroglaucin trimethyl ether. Hence it follows that erythroglaucin must be a monomethyl ether of catenarin and since catenarin is soluble in dilute aqueous Na_2CO_3 and in dilute aqueous ammonia while erythroglaucin is quite insoluble in these reagents it appeared probable that it is the hydroxyl group in position 7 in catenarin which appears as a methyl ether grouping in erythroglaucin. This conclusion, which is based on the well known and generally accepted belief that among the polyhydroxyanthraquinones only those possessing a hydroxyl group in a β position are soluble in dilute aqueous Na_2CO_3, has been confirmed by the conversion of catenarin into erythroglaucin. The possibility that catenarin contains a β-hydroxyl group was discussed by Raistrick *et al.* [1934], but was rejected, despite the solubility of catenarin in aqueous Na_2CO_3, since it was shown that catenarin is unaffected by heating for 8 hr. at 100° with methyl iodide and sodium methoxide in methanol. In this experiment a large excess of sodium was used, the proportions of reagents being catenarin (0·4 g.), methyl iodide (1 g. $\equiv$ 5·0 mol.) and sodium (1 g. $\equiv$ 31·0 mol. dissolved in methanol, 10 ml.). We have now found that if the proportions of reagents are altered to catenarin ($\equiv$1 mol.), methyl iodide ($\equiv$4 mol.) and sodium ($\equiv$4 mol.) erythroglaucin is formed in good yield. The same method was used for the conversion of *Frangula*-emodin (I, $R=H$) into its 7-methyl ether, physcion (I, $R=CH_3$) by Jowett & Potter [1903] and by Tutin & Clewer [1910]. There can thus be no reasonable doubt that erythroglaucin is the 7-methyl ether of catenarin and that it is in fact *1:4:5-trihydroxy-7-methoxy-2-methylanthraquinone*. Hence erythroglaucin (IV, $R=CH_3$) bears exactly the same relationship to catenarin (IV, $R=H$) as does physcion (I, $R=CH_3$) to *Frangula*-emodin (I, $R=H$).

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Experimental

Two highly purified specimens of catenarin were used during this work.

We prepared the first specimen from a commercial sample of catenarin, m.p. 230–232°, which was isolated from the mycelium of *Helminthosporium gramineum* Rabenhorst L.S.H.T.M. Cat. No. Ag 106 [Charles *et al.* 1933] and was kindly given to us by Mr M. Barrowcliff, research manager of British Dyestuffs Corporation Ltd. The original sample was purified by acetylation with acetic anhydride and conc. H_2SO_4, hydrolysis of the purified acetate with aqueous 2*N* NaOH in an atmosphere of nitrogen and crystallization from ethanol of the regenerated catenarin. The purified specimen formed large, glistening, deep red plates which melted sharply at 246° (Raistrick *et al.* [1934] give 246°). (Found, on material dried at 110°: C, 62·95; H, 3·55%. Calc. for $C_{15}H_{10}O_6$: C, 62·91; H, 3·52%.) On oxidation with chromic acid (Kuhn-Roth method) evidence of one side-chain methyl group was obtained. (Found: 102·4, 99·5% of 1 mol. CH_3COOH.)

The second specimen was prepared for us by our colleague Dr J. Breen by a much simpler method. The crude mixture of catenarin and helminthosporin (22·4 g.) obtained by ether and chloroform extraction of the dried mycelium (110 g.) of *Helminthosporium catenarium* Drechsler, L.S.H.T.M. Cat. No. Ag 127 [Raistrick *et al.* 1934] was vigorously stirred for 30 min. with cold 2% aqueous Na_2CO_3 (5½ litres). The deep purple solution was filtered from undissolved helminthosporin (3·8 g.), acidified with HCl and heated to flocculate the gelatinous precipitate of catenarin. This was filtered, washed and dried and gave pure catenarin (17·5 g.) after one crystallization from ethanol.

Reduction of catenarin. Formation of Frangula-*emodin*

A mixture of catenarin (1 g.), glacial acetic acid (20 ml.), HI (4 ml. sp.gr. 1·7) and red phosphorus (1 g.) was boiled under a reflux condenser for 5 hr. The mixture was cooled, poured into water (200 ml.) and decolorized by the addition of a slight excess of aqueous $NaHSO_3$. The product which separated (1·38 g.) was collected and extracted with boiling glacial acetic acid (150 ml.). The reduction product separated as pale yellow micro-crystalline rectangular plates (0·68 g.) which decomposed with intense blackening from 250° upwards and had all the other properties of emodin anthranol. This product (0·68 g.) was dissolved in boiling glacial acetic acid (150 ml.), the solution cooled to 60°, and a solution of CrO_3 (0·68 g.) in water (10 ml.) and acetic acid (10 ml.) was quickly added. The dark brown reaction mixture was held at 60° for 30 min. and then evaporated *in vacuo* to 25 ml. The cinnamon-coloured broad needles which separated were collected, washed well with acetic acid and dried (0·35 g.; m.p. 253–254°). On crystallization from toluene (norite) it was obtained as fine, dull orange needles (0·23 g.; m.p. 257°). A mixture with authentic *Frangula*-emodin (m.p. 254–255°) melted at 255–256°. (Found: C, 66·60; H, 3·60%. Calc. for $C_{15}H_{10}O_5$: C, 66·64; H, 3·73%.) Specimens of the product from catenarin and of *Frangula*-emodin gave identical reactions with the following reagents: aqueous 2% $NaHCO_3$, insol.; 2% Na_2CO_3, red solution; 2*N* NaOH, red solution with a bluish shade; conc. H_2SO_4, red solution with a bluish shade; glacial acetic acid, pure yellow, non-fluorescent solution. These reactions are quite different from those given by catenarin which, *inter alia*, gives an orange solution with a marked greenish fluorescence in acetic acid.

The identity of the product from catenarin with *Frangula*-emodin was confirmed by conversion into the trimethyl ether. To a boiling solution of the

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product from catenarin (0·15 g.) in acetone (10 ml.) and dimethyl sulphate (3 ml.), 2*N* NaOH (17 ml.) was added in portions of 2–3 ml. After the addition of 12 ml. of NaOH the methylated product separated as a voluminous mass. The methyl ether was collected (0·18 g.) and crystallized first from ethyl acetate and then from acetone. Canary yellow rods, M.P. 225°, not depressed on admixture with authentic emodin trimethyl ether (M.P. 225°), prepared from *Frangula*-emodin by the same method. (Fischer *et al.* [1911], also give 225°.) (Found: C, 69·00, 69·16; H, 5·24, 5·18; CH_3O, 29·5, 29·7%. Calc. for $C_{18}H_{16}O_5$: C, 69·20; H, 5·17; $3CH_3O$, 29·8%.) The two products dissolve in conc. H_2SO_4 to give identical permanganate-coloured solutions which are quite different from, and much bluer in tone than, that given by emodin itself.

Oxidation of tetra-acetyl catenarin

The experimental conditions used were the same as those followed successfully by Anslow *et al.* [1940] for the oxidation of triacetyl emodin and tetra-acetyl ω-hydroxyemodin, a yield of 70–80% of triacetyl emodic acid being obtained in each of these cases. Tetra-acetyl catenarin (1 g.) was dissolved in a mixture of glacial acetic acid (20 ml.) and acetic anhydride (20 ml.) at 50–60° and, while maintaining this temperature, a solution of chromic anhydride (2 g.) in water (1·6 ml.) and glacial acetic acid (20 ml.) was added drop by drop during 30 min. The temperature was then raised and held at 65–70° for 3 hr. The reaction mixture, which slowly became deep green in colour, was cooled, and poured into water (600 ml. at 50°), when there was immediate separation of pale yellow fine needles, which were insoluble in 2% aqueous $NaHCO_3$. Weight 0·39 g., M.P. 232°, mixed M.P. with authentic tetra-acetyl catenarin, 234–235°. It was confirmed that this material was unchanged tetra-acetyl catenarin by regenerating the parent hydroxyanthraquinone which, after crystallizing from ethanol, melted at 243–244° and did not depress the M.P. of pure catenarin, M.P. 246°. The green filtrate from the recovered tetra-acetyl catenarin was evaporated *in vacuo* to about 100 ml. and was exhaustively extracted with ether in a continuous extractor. On removal of the solvent a small amount of gummy material remained (0·09 g.) which was obviously not an anthraquinone carboxylic acid. Hence, although considerable oxidation had obviously taken place this did not result in the formation of the carboxylic acid corresponding to catenarin, as occurs with emodin, ω-hydroxyemodin and helminthosporin, but in extensive breakdown of the molecule as a whole.

Catenarin tetramethyl ether

Catenarin cannot be satisfactorily methylated with methyl sulphate and NaOH either in aqueous or in boiling acetone solution by the method which is very suitable for the methylation of emodin (see above). The following method was therefore used. Catenarin (1 g.) was dissolved in boiling acetone (50 ml.) and methyl sulphate (5 ml.) and anhydrous K_2CO_3 (5 g.) were added in 1 ml. and 1 g. portions at suitable intervals during 6 hr. boiling under reflux. The mineral salts were separated by filtration and were thoroughly extracted with acetone. Evaporation of the solvent gave 0·84 g. of *catenarin tetramethyl ether*, M.P. 189–190°, raised to 190–191°, by recrystallization from acetone from which it separated in golden-yellow leaflets. (Found: C, 66·49, 66·60; H, 5·28, 5·21; CH_3O, 36·1, 35·9%. $C_{19}H_{18}O_6$ requires C, 66·63; H, 5·30; $4CH_3O$, 36·3%.) Catenarin tetramethyl ether does not depress the M.P. of erythroglaucin trimethyl ether (Ashley *et al.* [1939], M.P. 187–188°). Both substances are insoluble in aqueous 2*N* NaOH and both give a deep pure blue solution in conc. H_2SO_4.

Oxidation of catenarin tetramethyl ether. Isolation of 4-methyl-3:6-dimethoxyphthalic anhydride and 3:5-dimethoxyphthalic anhydride

Catenarin tetramethyl ether (3 g.) was dissolved in a mixture of acetic anhydride (30 ml.) and glacial acetic acid (20 ml.). To this solution, held in a vigorously boiling water bath, there was added during 15 min. and with mechanical stirring, a solution of CrO_3 (7·2 g.) in water (4·8 ml.) and glacial acetic acid (30 ml.). The heating and stirring were continued for a further 15 min.

The dark green reaction mixture was cooled, evaporated *in vacuo* to a syrup (20–25 ml.) and held at 0° for 1 hr. The syrup, now containing crystals, was triturated with four successive 200 ml. amounts of dry ether. The combined ethereal extracts, separated from the green solid (A), were evaporated to low bulk and finally to 5 ml. *in vacuo*. On standing, crystals separated which were filtered, washed with acetic anhydride and then with ether and dried. Treatment of the combined mother liquors and washings (B) is described later. The crystals (0·23 g., M.P. 196–198°) were recrystallized from benzene in pale yellow needles, M.P. 202°, of *4-methyl-3:6-dimethoxyphthalic anhydride*. This specimen, a synthetic specimen (see below) and a mixture of the two, all melted at 202° ± 0·5°. The oxidation product also showed the same violet-blue fluorescence in benzene and green fluorescence in conc. H_2SO_4 as does the synthetic product. (Found, on the oxidation product: C, 59·61, 59·58; H, 4·62, 4·51; CH_3O, 27·9, 27·6 %. $C_{11}H_{10}O_5$ requires C, 59·43; H, 4·54; $2CH_3O$, 27·9 %.) On oxidation with chromic acid (Kuhn-Roth method) evidence of one side-chain CH_3 group was obtained, i.e. 102·7, 99·2 % of 1 mol. CH_3COOH. The dried green solid (A) was exhaustively extracted (Soxhlet) with ether to give 0·17 g. of a yellow solid from which, after sublimation in a high vacuum followed by crystallization from benzene, a further 0·07 g., M.P. 199–201°, of 4-methyl-3:6-dimethoxyphthalic anhydride was obtained. Total yield, 0·30 g.

The combined mother liquors and washings (B) were evaporated to dryness *in vacuo* to yield a gum from which, by trituration with ether followed by crystallization from benzene, 0·12 g. of unchanged catenarin tetramethyl ether, M.P. 186–188°, was separated. The mother liquors from this substance were dried and sublimed in a high vacuum to give 0·18 g. of a yellow micro-crystalline sublimate with an indefinite M.P. and consisting essentially of a mixture of 3:5-dimethoxyphthalic anhydride and 4-methyl-3:6-dimethoxyphthalic anhydride. These two substances were separated by dissolving the mixture in hot 2*N* NaOH (3 ml.) and acidifying the cooled solution with HCl. 3:5-Dimethoxyphthalic acid separated first in thick compact prisms (0·09 g.) which are readily distinguished from 4-methyl-3:6-dimethoxyphthalic acid which separated later in fine thin needles. The 3:5-dimethoxyphthalic acid was heated for a few minutes at 170° when it melted and gave off water. The resulting 3:5-dimethoxyphthalic anhydride was purified by sublimation in a high vacuum. It was thus obtained in colourless micro-needles (0·06 g.). This specimen, an authentic specimen of 3:5-dimethoxyphthalic anhydride and a mixture of the two all melted at 147–148° (lit. 147°). (Found: C, 57·73, 57·82; H, 3·87, 3·90; CH_3O, 29·9, 29·5 %. $C_{10}H_8O_5$ requires C, 57·68; H, 3·88; $2CH_3O$, 29·8 %.)

Synthesis of 4-methyl-3:6-dimethoxyphthalic anhydride

(*a*) *2:5-Dihydroxy-3:4-dicyanotoluene.* To a solution of toluquinone (10 g.) in ethanol (300 ml.), cooled in ice, conc. H_2SO_4 (12·5 ml.) in ethanol (25 ml.) was added. A solution of KCN (30 g.) in water (60 ml.) was cautiously added, with vigorous stirring, until the mixture became brown in colour and alkaline

in reaction. The mixture was then made slightly acid by the addition of alcoholic H_2SO_4 and after 1–2 hr. was evaporated *in vacuo* to remove the ethanol and excess HCN. The sticky residue, consisting of a mixture of *2:5-dihydroxy-3:4-dicyanotoluene*, toluquinol and K_2SO_4, was diluted with water (500 ml.) and the dark tarry matter which separated (0·27 g.) was filtered off and discarded. The filtrate was extracted with ether and the dark crystalline residue (11–12 g.) remaining on removal of the solvent and consisting of a mixture of 2:5-dihydroxy-3:4-dicyanotoluene and toluquinol was ground with three 100 ml. lots of cold chloroform which removed the toluquinol, the crude 2:5-dihydroxy-3:4-dicyanotoluene remaining as a buff-coloured powder (33·2 g. from 60 g. of toluquinone = 77·6 % theor.).

2:5-Dihydroxy-3:4-dicyanotoluene cannot be satisfactorily crystallized from any of the customary solvents but is readily characterized by its crystalline diacetate and dimethyl ether (see below). A specimen was purified for analysis by fractional sublimation in a high vacuum at 140–150° and was so obtained in pale yellow micro-crystals which have no definite M.P. but darken from 195° and become completely black at about 240°. (Found: C, 61·88; H, 3·42; N, 16·1 %. $C_9H_6O_2N_2$ requires C, 62·05; H, 3·48; N, 16·1 %.) The substance is readily soluble in ethanol, ethyl acetate, ether and acetone but is almost insoluble in chloroform, benzene and toluene. Unlike the corresponding benzene compound it is fairly readily soluble in cold water and aqueous solutions give the following reactions: (*a*) With $FeCl_3$, a violet colour changing to brown on standing. (*b*) With dilute NaOH, the colourless solution, which shows a blue fluorescence, becomes yellow with an intense green fluorescence.

(*b*) *2:5-Diacetoxy-3:4-dicyanotoluene.* Crude 2:5-dihydroxy-3:4-dicyanotoluene (2 g.) was dissolved in a mixture of acetic anhydride (20 ml.) and conc. H_2SO_4 (1 ml.). The acetylation mixture, after standing for a short time, was poured into iced water and the precipitated acetate (2·3 g.) was crystallized from ethanol. *2:5-Diacetoxy-3:4-dicyanotoluene* crystallizes in long colourless rods, M.P. 128°. (Found: C, 60·31; H, 4·00; N, 10·85 %. $C_{13}H_{10}O_4N_2$ requires C, 60·43; H, 3·90; N, 10·85 %.) Solutions of the substance in benzene and still more in ethanol show, in the light from a mercury vapour lamp, a blue fluorescence which is not visible in daylight.

(*c*) *2:5-Dimethoxy-3:4-dicyanotoluene.* The crude mixture (11–12 g.) of 2:5-dihydroxy-3:4-dicyanotoluene and toluquinol from toluquinone (10 g.) (see section (*a*), above) was dissolved in acetone (150 ml.) and methyl sulphate (30 ml.). Aqueous 2*N* NaOH (150 ml.) was added in portions to the solution which was boiled for 30 min. *2:5-Dimethoxy-3:4-dicyanotoluene* crystallized from the cooled mixture and was separated from the oily toluquinol dimethyl ether by filtration. It was washed with chilled 50 % aqueous acetone (100 ml.) followed by glacial acetic acid (50 ml.) and was so obtained as colourless needles (5·5 g.; M.P. 182°) which may be converted into the anhydride without further purification. It crystallizes with very little loss from glacial acetic acid in long colourless needles, M.P. 182°. (Found: C, 65·13; H, 4·90; N, 13·8; CH_3O, 30·4 %. $C_{11}H_{10}O_2N_2$ requires C, 65·31; H, 4·99; N, 13·9; $2CH_3O$, 30·7 %.) Solutions of the substance in ethyl acetate, glacial acetic acid, ethanol and benzene are colourless with no apparent fluorescence in daylight but all show a faint blue fluorescence in the light from a mercury vapour lamp.

(*d*) *4-Methyl-3:6-dimethoxyphthalic anhydride.* 2:5-Dimethoxy-3:4-dicyanotoluene (2 g.) was dissolved in 20 ml. of a mixture of conc. H_2SO_4 (200 ml.) and water (20 ml.) and hydrolysed by heating in a boiling water bath for 20 min. The solution quickly became orange-yellow in colour with a strong green

fluorescence. It was cooled and poured into water (20 ml.) when there was a copious separation of pale yellow needles from the hot solution. A further 20 ml. of water were added, the mixture cooled and held at 0° for 2 hr. The crystals were separated by filtration, washed well with cold water and dried. Weight of almost pure anhydride from six such experiments, i.e. from 12 g. of dinitrile, 6·1 g. The yield of anhydride decreased on longer hydrolysis and was very small after 1·5 hr. (0·1 g. from 2 g.). The filtrate and washings, on evaporation on a boiling water bath, gave 3·2 g. of a mixture of crystalline substances from which a further 2·3 g. of the anhydride were isolated by extraction with aqueous 5% $NaHCO_3$ and fractional crystallization from benzene. *4-Methyl-3:6-dimethoxyphthalic anhydride* crystallizes well from glacial acetic acid or benzene in long pale yellow needles, M.P. 202°, which readily sublime in a high vacuum at 135°. (Found: C, 59·56; H, 4·49; N, nil; CH_3O, 27·9%. $C_{11}H_{10}O_5$ requires C, 59·43; H, 4·54; $2CH_3O$, 27·9%.) The substance does not dissolve in cold 2*N* NaOH except on long standing, but quickly dissolves on heating. Dilute solutions in ethyl acetate, ethanol, glacial acetic acid or benzene have a strong violet-blue fluorescence. Solutions in cold conc. H_2SO_4 are yellow, with a strong green fluorescence.

Methylation of catenarin with methyl iodide and sodium methoxide. Formation of erythroglaucin

A mixture of catenarin (1·144 g.≡1/250 mol.), methyl iodide (2·272 g.≡4/250 mol.) dissolved in methanol (10 ml.), and 10 ml. of a solution of Na (1·84 g.) in methanol (50 ml.), i.e. 0·368 g. Na≡4/250 mol., was heated in a sealed glass tube at 100° for 4 hr. The brownish purple reaction mixture was poured into water (200 ml.), acidified with HCl, filtered, washed and dried. The crude reaction product (1·09 g.) was crystallized from glacial acetic acid (100 ml.+norite) and gave 0·56 g. of almost pure *catenarin 7-methyl ether*, M.P. 197–200°. This material was recrystallized several times from glacial acetic acid to constant M.P. Pure catenarin 7-methyl ether was finally obtained in this way as large shining bright red plates, M.P. 205–206°. An authentic specimen of erythroglaucin, isolated from *Aspergillus ruber* (Spieckermann and Bremer) Thom and Church, L.S.H.T.M. Cat. No. A. 22, by Ashley *et al.* [1939] and recrystallized from acetic acid, melted at 205–206°, and a mixture with catenarin 7-methyl ether melted at the same temperature. (Found, on catenarin 7-methyl ether: C, 63·85, 63·93; H, 4·00, 4·08; CH_3O, 10·05, 10·1%. $C_{16}H_{12}O_6$ requires C, 63·98; H, 4·03; CH_3O, 10·3%.)

Catenarin 7-methyl ether and natural erythroglaucin, compared side by side, gave identical reactions with the following reagents. (*a*) Glacial acetic acid, an orange-red solution with a green fluorescence. (*b*) Conc. H_2SO_4, a solution which is pure blue in colour in very dilute solution but becomes a deep violet-blue by transmitted light in more concentrated solutions. (*c*) Aqueous *N* NaOH. The red crystals immediately become dark purple in colour and slowly dissolve to a permanganate-coloured solution which, on standing, slowly deposits a violet precipitate. (*d*) Aqueous 2% $NaHCO_3$, aqueous 2% Na_2CO_3 or aqueous 2*N* NH_4OH, quite insoluble. The behaviour towards aqueous Na_2CO_3 and aqueous NH_4OH is in marked contrast to that of catenarin which dissolves in 2% Na_2CO_3 giving a cerise solution and in 2*N* NH_4OH to a reddish violet solution.

Triacetylcatenarin-7-methyl ether. Catenarin-7-methyl ether (0·08 g., M.P. 205–206°) was dissolved in 4 ml. of a mixture of pyridine (10 ml.) and acetic anhydride (2 ml.) and held at 37° for 24 hr. The acetate (0·09 g.), which was

precipitated on pouring the acetylation mixture into water, was crystallized from ethanol. Yellow rods, M.P. 229–230°. (Found: C, 62·22, 62·01; H, 4·45, 4·12; CH_3O, 7·3, 7·1%. $C_{22}H_{18}O_9$ requires C, 61·95; H, 4·26; CH_3O, 7·3%.) Triacetylerythroglaucin, prepared similarly, melted at 230–231° and a mixture of the two substances at 230–231°.

Summary

The molecular constitutions of catenarin and erythroglaucin have been determined.

Catenarin, $C_{15}H_{10}O_6$, which constitutes over 15% of the dry weight of the mycelium of *Helminthosporium catenarium* Drechsler, gives *Frangula*-emodin, 4:5:7-trihydroxy-2-methylanthraquinone, on reduction with HI and red phosphorus, followed by oxidation with chromic acid of the resulting anthranol. *Catenarin tetramethyl ether* on oxidation with chromic acid yields a mixture of *4-methyl-3:6-dimethoxyphthalic anhydride* (the synthesis of which is described) and 3:5-dimethoxyphthalic anhydride. These, and other experimental facts previously established, determine the constitution of catenarin as *1:4:5:7-tetrahydroxy-2-methylanthraquinone.*

Erythroglaucin, $C_{16}H_{12}O_6$, a metabolic product of species in the *Aspergillus glaucus* series, has been shown to be the 7-methyl ether of catenarin, i.e. *1:4:5-trihydroxy-7-methoxy-2-methylanthraquinone.* It was prepared, in good yield, by methylating catenarin with methyl iodide and sodium methoxide in methanol solution.

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135. INHIBITION OF THE SUCCINOXIDASE SYSTEM BY EXTRACTS OF TUMOUR AND NORMAL TISSUES

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(*Received 8 April 1940*)

THE "succinoxidase" activity of certain tumour tissues was found by Elliott *et al.* [1935] and Elliott & Greig [1938] to be very low. It seemed possible that the low activity might be due to the presence in the tumours of an inhibitor of the system as well as to deficiency of the enzymes and carriers responsible for succinate oxidation.

In testing this possibility it was found that the oxidation of succinate by liver suspensions was rapidly and almost completely inhibited by the presence of suspensions or extracts of Walker 256 carcinoma and Philadelphia No. 1 sarcoma, and less actively by Jensen sarcoma. Inhibitory effects were also shown by a number of normal tissues, especially pancreas, which have low succinoxidase activity. Since heart, liver, and kidney, are extremely active in succinoxidase, it was not possible to determine whether they also contain some of the inhibitor. However, incubation of a concentrated liver suspension at 38° for 30 min. before diluting it and determining its activity, caused no change in its activity, which indicated that there was little inhibitor present.

When testing the inhibitory activity of tissue preparations which themselves show succinoxidase activity, it was assumed that their succinoxidase activity was not affected by the presence of small amounts of liver. If A was the O_2 uptake due to liver alone, B that due to the other tissue preparation alone, and C the O_2 uptake with both together, the percentage inhibition of liver activity by the other tissue preparation was taken as $\frac{A+B-C}{A}100$. Most of the work on the inhibitor was done with suspensions and extracts of Walker 256 carcinoma since this tissue was conveniently available and contained a very active inhibitor.

The inhibitor acted on the succinoxidase in a progressive manner, the inhibition increasing with time, and it appeared that its action involved a destruction of catalytic components of the succinoxidase system. The inhibition of succinate oxidation was more pronounced from the start if liver suspension was first incubated with tumour extract or suspension before adding the succinate.

The inhibitor could be obtained in an opalescent solution by centrifuging phosphate buffer suspensions of homogenized or sand-ground tissues. After dialysis overnight in narrow cellophane bags against distilled water, none of the inhibitor seemed to be lost. Heating to 100° for 10 min. caused considerable delay in the inhibitory activity of the extracts but did not abolish it. When tumour slices instead of suspension or extract were used, the inhibitory effect, as might be expected, was initially quite low but increased steadily as the inhibitor diffused out into the medium.

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Pancreas extracts were very strongly inhibitory and the inhibitory activity of tissue extracts could be imitated with dilute solutions of commercial trypsin (Difco). It seems likely that the inhibitor is a proteolytic enzyme which destroys components of the succinate oxidizing system.

Results showing the above effects are given in Tables 1 and 3.

Table 1. *Summary of effects of tissue suspensions and extracts on the rate of succinate oxidation by other tissue suspensions*

Tissue *A*	Moist wt. mg.	Tissue *B*	Moist wt. mg.	% inhibition of *A* by *B* (assuming no effect of *B* on *A*). 0–30 min.	30–60 min. (30–45 min.)*	60–90 min.
Liver suspension (rat)	50	Walker 256 carc. suspension	200	87	97	—
,, (rabbit)	50	,, carc. suspension	200	90	99	—
,, (rat)	50	,, carc. suspension	190	63	(89)	—
,, ,,	50	,, extract from	190	72	(94)	—
,, ,,	50	,, extract dialysed from	190	63	(90)	—
,, ,,	50	,, extract heated 6 min. at 100°	190	35	(61)	—
,, ,,	50	,, suspension	200	85	96	98
,, ,,	50	,, suspension	100	78	93	97
,, ,,	50	,, suspension	50	63	86	93
,, ,,	50	,, suspension	50	—	—	—
(Incubated separately and together 30 min. before adding succinate)			—	82	91	95
Liver suspension	50	Walker 256 slices	350	35	66	88
,,	25	Philadelphia No. 1 sarc. suspension	200	78	87	—
,,	25	,, extract dialysed from	200	86	91	—
,,	25	,, extract heated 10 min. at 100°	200	9	40	—
,,	50	Jensen sarc. suspension	200	36	66	—
,,	25	,, suspension	200	25	80	—
,,	25	,, extract dialysed from	180	79	88	—
,,	50	Pancreas suspension	200	92	97	—
,,	50	Spleen suspension	200	67	75	71
,,	50	,, ,,	200	—	—	—
(Incubated separately and together 30 min. before adding succinate)			200	72	70	72
Liver suspension	25	Spleen extract	200	32	80	86
,,	25	Muscle suspension	200	16	(20)	—
,,	25	Brain suspension	200	29	54	—
,,	25	Brain extract dialysed from	180	5	9	—
,,	50	Testis suspension	200	18	26	—
,,	50	Thymus suspension	200	39	56	—
,,	50	Lung suspension	200	29	71	—
,,	50	Blood	200	−11†	−19†	—
,,	50	Trypsin (Difco) solution	30	82	95	—
,,	50	,,	9	57	88	—
Muscle suspension	200	Walker 256 suspension	190	70	(73)	—
Brain suspension	200	Philadelphia No. 1 suspension	200	20	47	—
Heart suspension	25	Walker 256 extract from	200	9	6	4
Kidney suspension	25	Walker 256 extract from	200	89	96	—

* Figures in brackets for 15 min. only, after first 30 min. † Acceleration.

It is now generally accepted that the succinoxidase system consists of at least three catalysts, succinic dehydrogenase, cytochrome, and cytochrome oxidase. The dehydrogenase causes succinate to reduce cytochrome, and the reduced cytochrome is reoxidized by cytochrome oxidase. It has recently become apparent that an extra catalyst is interposed between the dehydrogenase and

cytochrome [Hopkins *et al.* 1939; Horecker *et al.* 1939; Stern & Melnick, 1939; Euler & Hellström, 1939].

Attempts were made to discover which particular component of the system was specially inhibited. The activity of liver succinic dehydrogenase, determined by methylene blue reduction, was considerably inhibited (50–60 %, Table 2) by the presence of tumour extract, but the inhibition was not as complete as was the case with the aerobic succinate oxidation, and previous incubation of the liver suspension with the tumour extract did not increase the inhibition.

When cytochrome *c* was added to the liver suspension the rate of O_2 uptake with succinate was considerably increased. Tumour extract caused considerable inhibition of this O_2 uptake rate (Table 3). However, the rate in the presence of tumour extract was considerably greater when cytochrome *c* was added than when no cytochrome was added, indicating again that the effect of the inhibitor on the original succinoxidase activity was not due solely or mainly to action on the dehydrogenase. The % inhibition was less in the presence of added cytochrome *c* than in its absence.

Table 2. *Effect of extract of Walker No. 256 sarcoma on liver succinic dehydrogenase*

	Reduction time: No succ. t	Reduction time: With succ. $t_{succ.}$	Activity $600\frac{t-t_{succ.}}{t.t_{succ.}}$* ($\mu$l. O_2 equiv./hr.)	Inhibition of liver activity by tumour extract
Liver suspension, 35 mg.	∞	12	50	
Tumour extract, equiv. 200 mg.	196	100	2·8	
Liver + tumour extract	43	17	21·4	*c.* 63 %
After incubation of liver suspension, tumour extract, and the two mixed, for 60 min. at 38°.				
Liver suspension	∞	19	31·6	
Tumour extract	450 ±	260 ±	1·0	
Liver + tumour extract	95	25·5	17·0	*c.* 49 %

The same extract with the same liver suspension in the same amounts and at the same time, in an O_2 uptake experiment caused 53 % inhibition in the first 20 min., 75 % after 30 min., and 83 % after 60 min.

* Elliott & Greig [1938].

The activity of the liver cytochrome oxidase was determined by measuring the rate of O_2 uptake with *p*-phenylenediamine in the presence and absence of added cytochrome *c*. *p*-Phenylenediamine reduces oxidized cytochrome and the activity of the cytochrome oxidase-cytochrome system can be determined by measuring O_2 uptake in the presence of excess of *p*-phenylenediamine; when excess of cytochrome *c* as well as the diamine is added, a measure of the cytochrome oxidase itself is obtained. Tumour extracts themselves caused fairly rapid oxidation of *p*-phenylenediamine, especially when cytochrome *c* was added. As in the determination of the inhibition of succinate oxidation, it was assumed that the liver tissue did not affect the activity of the inhibitory extract.

Tumour extract inhibited the oxidation of *p*-phenylenediamine by liver considerably, but the extent of inhibition and the rate of increase of inhibition were less than with the succinate oxidation. In the presence of added cytochrome, the rate of *p*-phenylenediamine oxidation by liver suspension was much increased and tumour extract caused some inhibition which did not increase greatly with time. The rate of *p*-phenylenediamine oxidation by liver suspension with added cytochrome, even in the presence of tumour extract, was much greater than the rate without cytochrome, even in the absence of inhibitor. The

Table 3. *Effects of tissue extracts on succinate oxidation by liver and other tissue suspensions in the presence and absence of added cytochrome* c

Tissue *A*	Moist wt. mg.	Tissue *B*	Moist wt. mg.	O_2 uptake (μl.) 0–30 min. —	0–30 min. Cyt.	30–60 min. —	30–60 min. Cyt.	60–90 min. —	60–90 min. Cyt.	% inhibition of *A* by *B* (assuming *B* unaffected by *A*) 0–30 min. —	0–30 min. Cyt.	30–60 min. —	30–60 min. Cyt.	60–90 min. —	60–90 min. Cyt.
Liver suspension	50			401	537	289	397	—	—	—	—	—	—	—	—
		Walker 256 extract from	200	4	8	0	4	—	—	—	—	—	—	—	—
		Both together		52	234	11	123	—	—	87	58	96	70	—	—
Liver suspension	25			140	307	122	260	93	206	—	—	—	—	—	—
		Trypsin solution	15	0	0	0	0	0	0	—	—	—	—	—	—
		Both together		80	77	22	17	3	8	43	75	82	94	97	96
Liver suspension	25			183	309	143	260	123	212	—	—	—	—	—	—
		Spleen extract from	200	—	76	—	74	—	66	—	—	—	—	—	—
		Both together		125	344	29	284	17	232	32	13	80	19	86	22
Liver suspension	25			175	320	151	271	125	223	—	—	—	—	—	—
		Pancreas extract from	100	—	2	—	1	—	1	—	—	—	—	—	—
		Both together		12	23	3	2	0	3	93	93	98	100	100	99
Liver suspension	25			74	204	61	179	—	—	—	—	—	—	—	—
		Pancreas extract from	25	—	2	—	0	—	—	—	—	—	—	—	—
		Both together		20	26	2	4	—	—	73	88	97	98	—	—
Heart suspension	25			192	267	162	208	142	190	—	—	—	—	—	—
		Walker 256 extract from	180	—	53	—	45	—	45	—	—	—	—	—	—
		Both together		174	443	152	360	136	293	9	−46*	6	−51*	4	−32*
Kidney suspension	20			128	343	97	293	—	—	—	—	—	—	—	—
		Walker 256 extract from	190	—	17	—	9	—	—	—	—	—	—	—	—
		Both together		14	110	4	70	—	—	89	73	96	79	—	—
Walker 256 suspension	200			15	119	9	96	7	76	—	—	—	—	—	—
Walker 256 slices	350			31	—	15	—	8	—	—	—	—	—	—	—
Walker 256 slices	300			—	63	—	56	—	54	—	—	—	—	—	—

* Acceleration.

Table 4. *Effects of tissue extracts on* p-*phenylenediamine oxidation by liver and other tissue suspensions in the presence and absence of added cytochrome* c

Tissue *A*	Moist wt. mg.	Tissue *B*	Moist wt. mg.	O_2 uptake (μl.) 0–30 min. —	0–30 min. Cyt.	30–60 min. —	30–60 min. Cyt.	60–90 min. —	60–90 min. Cyt.	% inhibition of *A* by *B* (assuming *B* unaffected by *A*) 0–30 min. —	0–30 min. Cyt.	30–60 min. —	30–60 min. Cyt.	60–90 min. —	60–90 min. Cyt.
Blank, no tissue*				9	15	16	27	17	27	—	—	—	—	—	—
Liver suspension	50			165	—	133	—	122	—	—	—	—	—	—	—
		Walker 256 extract from	200	29	—	27	—	28	—	—	—	—	—	—	—
		Both together		114	—	79	—	60	—	49	—	61	—	74	—
After 45 min. incubation before adding substrate															
Liver				121	—	—	—	—	—	—	—	—	—	—	—
		Walker 256		33	—	—	—	—	—	—	—	—	—	—	—
		Both together		67	—	—	—	—	—	72	—	—	—	—	—
Liver suspension	10			—	224	—	165	—	139	—	—	—	—	—	—
		Walker 256 suspension	100	—	377	—	286	—	207	—	—	—	—	—	—
		Both together		—	526	—	373	—	255	—	34	—	47	—	65
After 40 min. incubation before adding substrate															
Liver				—	234	—	177	—	—	—	—	—	—	—	—
		Walker 256		—	342	—	235	—	—	—	—	—	—	—	—
		Both together		—	533	—	337	—	—	—	18	—	42	—	—
Liver suspension	20			89	330	71	241	—	—	—	—	—	—	—	—
		Walker 256 extract from	200	38	120	40	78	—	—	—	—	—	—	—	—
		Both together		65	295	64	203	—	—	70	47	66	48	—	—
Liver suspension	25			—	457	—	339	—	265	—	—	—	—	—	—
		Pancreas extract from	100	—	22	—	0	—	3	—	—	—	—	—	—
		Both together		—	66	—	20	—	12	—	90	—	94	—	97
Liver suspension	25			—	431	—	309	—	237	—	—	—	—	—	—
		Spleen extract from	200	—	272	—	167	—	97	—	—	—	—	—	—
		Both together		—	624	—	363	—	219	—	18	—	37	—	49
Liver suspension	25			—	544	—	400	—	308	—	—	—	—	—	—
		Trypsin	15	—	1	—	4	—	9	—	—	—	—	—	—
		Both together		—	227	—	151	—	103	—	58	—	63	—	69
Heart suspension	25			345	—	276	—	224	—	—	—	—	—	—	—
		Walker 256 extract from	200	29	—	27	—	28	—	—	—	—	—	—	—
		Both together		173	—	122	—	76	—	58	—	66	—	79	—
Heart suspension	10			—	259	—	184	—	136	—	—	—	—	—	—
		Walker 256 extract from	180	—	241	—	194	—	150	—	—	—	—	—	—
		Both together		—	513	—	374	—	265	—	−5	—	2	—	15
Kidney suspension	10			—	267	—	233	—	—	—	—	—	—	—	—
		Walker 256 extract from	200	—	105	—	55	—	—	—	—	—	—	—	—
		Both together		—	153	—	62	—	—	—	82	—	97	—	—

* The blank O_2 uptakes have been deducted from all observed O_2 uptakes.

rate of *p*-phenylenediamine oxidation depends upon both the concentration of oxidase and of cytochrome *c* [Elliott & Greig, 1938] and, while the extract evidently inhibited some of the oxidase, the active remainder of the oxidase could give a larger O_2 uptake when excess of cytochrome was added. But since the inhibition of cytochrome oxidase was less extensive than the inhibition of the "succinoxidase", cytochrome oxidase inhibition could not fully account for the strong and rapidly progressive inhibition of the oxidation of succinate.

Cytochrome *c* did not appear to be affected at all by tumour extract; the intensity of its absorption bands, after reduction, and its catalytic activity were not affected by incubation with the extracts.

Since the intense progressive inhibition of the succinoxidase system of liver cannot be fully accounted for by destruction of succinic dehydrogenase, cytochrome *c*, or cytochrome oxidase, it seems that some other factor concerned in the succinoxidase system in liver must be strongly affected by the inhibitor. This can scarcely be the intermediate, discovered by Hopkins *et al.* [1939], which is necessary for cytochrome *c* reduction by succinate with succinic dehydrogenase, since addition of cytochrome *c* considerably increased succinate oxidation even in the presence of the inhibitory extract. It is possible that the factor affected is cytochrome *b*. It is known [Keilin, 1929; Ball, 1938] that succinate with its dehydrogenase can reduce cytochromes *a* and *b* as well as *c*, presumably through the intermediate of Hopkins *et al.* It is believed by many that these three cytochromes act in series, H being transferred from one to the next in the order *b*, *c*, *a*. However, the H from the succinic dehydrogenase system may reduce any cytochrome directly. If liver suspension is rich in cytochrome *b* but low in *c*, the effect of the inhibitor may be to destroy cytochrome *b*, thus cutting down the O_2 uptake, but the addition of cytochrome *c* may provide an alternative mediator of H transport. The same argument might suggest that cytochrome *a* is affected, but it now seems probable that cytochrome *a*, or at least one form of it (a_3), can be identified with cytochrome oxidase [Keilin & Hartree, 1939], and, as mentioned above, destruction of the oxidase cannot account for the whole effect of the inhibitor on liver succinoxidase.

The effects of spleen extract on the activity of liver enzymes were similar to, but less marked than, those of tumour extract. Pancreas extract was extremely active; small amounts of extract strongly inhibited all the activities measured. Trypsin in small amounts also inhibited all the activities though, as with the tumour inhibitor, the effect on cytochrome oxidase was not as great or progressive as the effect on succinate oxidation.

Tumour suspension inhibited the succinoxidase activity of rabbit liver quite as strongly as that of rat liver. The succinoxidase activities of rat brain and skeletal muscle suspension were appreciably inhibited by tumour suspensions. Rat kidney suspensions were strongly inhibited, the inhibition of *p*-phenylenediamine oxidation being about as strong as that of succinate oxidation.

The succinoxidase activity of heart suspensions was scarcely at all inhibited, and, in the presence of added cytochrome *c*, tumour extracts actually caused considerable acceleration (40–55%), allowance being made for the activity of the extracts themselves. The *p*-phenylenediamine oxidation by heart was inhibited appreciably in the absence of added cytochrome *c*, scarcely at all in its presence. It would seem that succinate oxidation in heart tissue is not mediated by the factor which is specially susceptible to the inhibitor. The extra acceleration of succinate oxidation by the tumour extract when cytochrome *c* was added is difficult to account for; it may possibly be due to a better dispersion of heart tissue by proteolytic enzymes in the extract. The inhibitor evidently

Both together 153 62 82 97
* The blank O_2 uptakes have been deducted from all observed O_2 uptakes.

affects some part of the cytochrome system of heart since, in the absence of added cytochrome *c*, the oxidation of *p*-phenylenediamine is considerably inhibited.

It is possible that the low succinoxidase activity found in some tumours and other tissues can be partly accounted for by the action of the inhibitor liberated under the experimental conditions. The rate of succinate oxidation by tumour suspensions and slices falls off very rapidly to a low value, but addition of cytochrome *c* increases the rate considerably, indicating that dehydrogenase and oxidase are present in fair amount all the time but that an intermediary is lacking (see bottom of Table 3).

This work emphasizes the need for caution in drawing conclusions as to the quantitative distribution of enzyme systems and their biochemical role from work on "surviving" whole tissue preparations. The nature and distribution of the inhibitor, and the nature of the particular mediator of succinate oxidation which seems to be most strongly affected, require further study.

In the paper of Elliott & Greig [1938], it was mentioned that, while the succinate-oxidizing activities of suspensions of a number of tissues were increased by adding cytochrome *c*, the activities of liver and kidney were not so affected. In the present work, rather low values for the succinoxidase activity of these two tissues were found without added cytochrome, and a considerable acceleration on adding cytochrome *c*. It is not understood why this was not previously observed.

Methods

The methods used were the same as those of Elliott & Greig [1938]. The fresh, weighed tissue was homogenized in *M*/20 phosphate buffer, *p*H 7·4, using the apparatus of Potter & Elvehjem [1936]. Occasionally the suspensions were prepared by grinding with sand. The extracts were obtained by centrifuging the suspensions clear, each extract being made up to the original volume of the suspension with a little buffer with which the sediment had been washed. Rat tissues were used throughout except for one experiment (in Table 1) with rabbit liver.

For the manometric experiments, the Barcroft manometer vessels contained air and a total of 3 ml. of fluid made up of the tissue suspensions, or extracts, substrate solution, and water, with a final concentration of *M*/30 phosphate buffer. The concentration of succinate used was 9 mg./ml., of *p*-phenylenediamine 10 mg./ml. When cytochrome *c* was added, 0·2 ml. of a solution containing 26 μg. bound Fe/ml. in 1 % NaCl were used, the solution being obtained by the method of Keilin & Hartree [1937]. In succinate experiments the two manometer flasks received the same additions except that succinate was put only in the right-hand flask. In *p*-phenylenediamine experiments, the left-hand flask contained only water; O_2 uptakes were corrected for the small, slightly autocatalytic oxidation of *p*-phenylenediamine which occurred in the absence of tissue and which was slightly increased in the presence of cytochrome solution.

The methylene blue reduction experiments were made as described by Elliott & Greig [1938] using bent Thunberg tubes shaken in a water bath. The 3 ml. of fluid contained liver suspension, tumour extract, 30 mg. neutralized succinic acid, and 0·5 ml. of 0·0703 %[1] methylene blue, and the reduction time was taken as the period required for the colour to match that in a tube containing the same materials, aerated, with 1/20 the amount of dye. The rate of dye reduction by succinic dehydrogenase was calculated by the formula used by Elliott & Greig which allows for the reduction time in the absence of succinate.

[1] In the paper of Elliott & Greig [1938] the concentration of methylene blue was incorrectly given as 0·703 %.

The succinic dehydrogenase activities of the liver suspension, tumour extract and the two mixed, were determined simultaneously. The inhibition of liver activity by tumour extract was calculated on the assumption that the liver material did not affect the activity of the tumour extract. Only extracts and not suspensions of tumour tissue could be used for these experiments since strong suspensions reduce methylene blue rapidly without succinate.

Summary

1. The aerobic oxidation of succinate with liver suspensions is strongly inhibited by suspensions or extracts of various tissues, especially of certain tumours, pancreas and spleen. The inhibitor is non-dialysable and, to some extent at least, thermolabile. Commercial trypsin in small amounts is also inhibitory.

2. The succinic dehydrogenase (anaerobic reduction of methylene blue with succinate) and the cytochrome oxidase (oxidation of *p*-phenylenediamine in the presence of added cytochrome *c*) are both inhibited by tumour extracts, but not strongly enough to account for the main effect of the inhibitor on the complete succinoxidase system of liver. Cytochrome *c* appears not to be affected. It is concluded that the inhibitor acts particularly strongly on some other component of the succinoxidase system of liver, possibly cytochrome *b*.

3. Kidney suspensions are affected similarly to liver suspensions. The succinoxidase activities of brain and muscle suspensions are also inhibited. But the succinoxidase activity of heart suspensions is scarcely at all affected and tumour extracts actually provoke an acceleration of succinate oxidation with heart suspension in the presence of added cytochrome *c*. The oxidation of *p*-phenylenediamine with heart is considerably inhibited by tumour extract in the absence of added cytochrome *c*, but not in its presence.

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136. FURTHER INVESTIGATIONS ON VITAMIN B_6 AND RELATED FACTORS OF THE VITAMIN B_2 COMPLEX IN RATS. PARTS I AND II

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(*Received 17 April 1940*)

RAPID advance in the biochemical analysis of the vitamin B_2 complex has not only led to the recognition, isolation and identification of an increasing number of food constituents of vitamin-like character, but has at the same time opened the way for the experimental production of diseases, the nutritional basis of which was hitherto unknown, hidden or barely suspected. The first approach to this systematic analysis resulted in the isolation of lactoflavin [György *et al.* 1933; Kuhn *et al.* 1933], now called riboflavin. The work was stimulated by clinical interest in dermatological conditions in which a nutritional cause was, possibly, in the background. The so-called "rat pellagra" of Goldberger and his co-workers [1926] has been regarded as one of the best examples of this group.

In the investigations leading to the isolation of riboflavin, growth-promoting activity had been used as a guide. In the search for more specific manifestations, it was soon established [György, 1934] that rat pellagra has no relationship to a lack of riboflavin but must be attributed to a deficiency caused by a different member of the vitamin B_2 complex, for which the term vitamin B_6 was proposed. This term has since then been generally accepted. Circumstantial evidence was offered [Birch *et al.* 1935] to the effect that the aetiology of rat pellagra is essentially different from that of human pellagra. Following the identification of nicotinic acid (nicotinic acid amide) with the preventive factor for pellagra in man and for blacktongue in dogs [Elvehjem *et al.* 1937], it became possible to state definitely that vitamin B_6 is, indeed, a separate member of the vitamin B_2 complex [Dann, 1937; György, 1937–8]. In order to avoid any further confusion, the term rat acrodynia was substituted [Birch *et al.* 1935] for rat pellagra. The revised designation emphasizes the outstanding characteristics of the cutaneous lesions caused by deficiency of vitamin B_6, such as the symmetrical dermatitis with oedema and scaliness, mainly on the peripheral parts of the body, for example, the mouth, nose, paws, ears and tail. Vitamin B_6 was defined, accordingly, as "that part of the vitamin B_2 complex which is responsible for the cure of the specific dermatitis developed by young rats fed on a vitamin B-free diet supplemented with purified vitamin B_1 and lactoflavin" [Birch & György, 1936]. In bioassays for vitamin B_6, cure of the acrodynia was used as the criterion, independently of the growth-promoting activity of the food constituents or concentrates tested.

In rats kept on a diet consisting of purified casein, melted butter fat, rice starch (or sucrose), salt mixture and cod liver oil, supplemented with vitamin B_1 and riboflavin, symptoms of vitamin B_6 deficiency appear in from 4 to 15 weeks,

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on an average in 7 weeks [György, 1935, 1]. These results have been confirmed in general by several authors who employed identical or similar diets. Dann [1939], however, reported that in an increasing number of his rats the specific cutaneous symptoms failed to develop and that he was unable, either by variation of the fat content of the diet or by using specially purified casein preparations, to increase the incidence of the rat acrodynia.

I. *Use of dried heated egg white as a constituent of the basal diet for the production of rat acrodynia*

Although in our laboratory the original diet proposed [György, 1935, 1] for the production of vitamin B_6 deficiency has proved, on the whole, very satisfactory, an attempt was made to improve the results with regard to frequency and time of appearance of rat acrodynia by the incorporation of dried egg white in the basal diet. In previous work [György, 1935, 1] it has been shown that egg white, although rich in riboflavin, is practically devoid of vitamin B_6. Thus, the addition of egg white could be expected to make the experimental diet more complete without furnishing it with vitamin B_6.

In view of the well-known injurious effect of raw egg white on rats, care was taken to add only relatively small amounts of egg white, detoxified by heat [Parsons & Kelly, 1933], to the experimental diet, in which casein remained the chief source of protein. In this connexion it should be pointed out, as a further safeguard against the possibly harmful effect of egg white, that casein contains the specific protective factor (vitamin H) for egg white injury in fairly high concentration [Boas, 1927].

Experimental

Rats at weaning were placed on the experimental ration under conditions that are now sufficiently well standardized to need no repeated description here. The basal diet had the following composition:

	%
Casein	18
Sucrose	68
Butter fat	8
Salt mixture [Hubbell *et al.* 1937]	4
Cod liver oil	2

Freshly separated egg whites were dried, either directly or, as was regularly done in later experiments, after they had been heated for 3 hr. at 80°, by placing them in shallow pans over a simmering water bath. The dry mass was then ground in a mortar, put through a sieve and incorporated in the diet at a level of 10%, replacing an equal quantity of sucrose. Two basal diets were thus obtained, differing only in that in one diet dried heated egg white replaced 10% of the sucrose. These diets will subsequently be referred to as the "no egg white diet" and the "10% egg white diet".

Each animal received a daily supplement of 20 μg. of synthetic vitamin B_1 and 20 μg. of synthetic riboflavin.[1] The temperature of the animal room was kept as constant as possible. The animals were weighed once a week.

Treatment with vitamin B_6 preparations of varying purity was begun when the symptoms became definite enough to warrant it. In reporting the results, correlations of weight and age with the incidence of acrodynia are made by comparison at the time of beginning the treatment rather than at the time of the

[1] Vitamin B_1 and riboflavin were supplied by Merck & Co. Inc., Rahway, New Jersey, and casein by General Biochemicals Inc., Cleveland, Ohio.

first appearance of the manifestations of acrodynia. In this manner questionable symptoms were obviated.

Post-mortem examinations were made on all rats that died without development of the symptoms of acrodynia, as soon after death as possible, in an attempt to discover intercurrent infections or concurrent deficiencies which might influence the incidence of acrodynia.

Results

A total of 519 rats from those kept on the two diets described was selected for the present investigation. Of this total, 328 were placed on the diet which contained no egg white, while 191 were placed on the diet containing 10 % egg white.

In 232 of the 328 rats placed on the "no egg white diet" (70·7 %), acrodynia developed of a severity intense enough to require treatment or to have been the cause of death before treatment was instituted. The average age of the rats in this group at the time treatment was begun was 53·1 days, the range being between 27 and 103 days. These figures are in good agreement with those previously reported [György, 1935, 1]. Of the 94 animals that died without exhibiting specific signs of acrodynia, post-mortem examination revealed that pneumonia developed in 21, purpura, anaemia, or adrenal haemorrhage in 10 [György *et al.* 1937], hepatic injury in 3 [György & Goldblatt, 1939], 9 had cutaneous lesions which recent investigations [György *et al.* 1939] have shown to be the result of a deficiency of pantothenic acid, and 51 showed no gross pathological changes to which death could be ascribed (Table 1).

Of the 191 rats fed on the diet containing 10 % of egg white, acrodynia developed in 167, or 87·4 %. In this group, however, the average age at which treatment was begun was 39·2 days, which is less by 2 weeks than that in the first group. The fluctuation of age at treatment in the second group was between 14 and 112 days. Of the animals that died without showing signs of acrodynia, 4 had pneumonia, in 2 kidney abscesses developed, 2 had symptoms of deficiency of pantothenic acid and 16 were found to be free from internal changes to which death could be attributed. In this group there were seen no cases of hepatic injury, panmyelophthisis (anaemia, purpura) or adrenal haemorrhage (Table 1).

In order to avoid any false conclusion it is important to note that in the rats kept on the "10 % egg white diet" which were subsequently treated with vitamin B_6 (and pantothenic acid), panmyelophthisis was repeatedly observed at post-mortem examination, and even hepatic injury was seen in one animal. Thus the egg white diet seems to be devoid of the maturation factor of the bone marrow and probably does not contain the preventive factor for hepatic injury.

Definite correlations between the initial weight of the animals kept on the deficient diets and the incidence of acrodynia were not apparent (Table 2).

The incidence of acrodynia has also been analysed in relation to the weight of the animals at the time treatment was begun. The rats were divided into 6 groups: (1) those which showed no weight response, (2) those which showed a gain in weight until treatment was begun (or a maximum gain in the case of death without development of acrodynia) of 0–5 g., (3) 5–10 g., (4) 10–15 g., (5) 15–20 g., and (6) those which showed a weight gain of 20 g. or over. The corresponding relationship is shown in Fig. 1. In rats placed on the diet that contained 10 % egg white there was a greater tendency to gain weight before treatment, despite the fact that the average time until treatment was shorter by 2 weeks in this group than in the group placed on the diet without egg white.

Table 1. *Incidence of acrodynia in rats fed on diets deficient in vitamin B_6 and cause of death in rats on same diets in which acrodynia did not develop*

Diet containing	Total no. of rats	Rats with acrodynia		Age at which treatment began, days			No. of rats in which acrodynia did not develop, grouped by cause of death						No. of rats in which acrodynia did not develop still living
		No.	%	Av.	Min.	Max.	Pneumonia	Purpura, anaemia, adrenal haemorrhage	Hepatic injury	Kidney abscess	Filtrate factor deficiency	Undetermined	
No egg white	328	232	70·7	53·1	27	103	21	10	3	0	9	51	2
10% egg white	191	167	87·4	39·2	14	112	4	0	0	2	2	16	0

Table 2. *Relation of incidence of acrodynia to weight of rats at the time they were placed on diets deficient in vitamin B_6*

Diet containing	Wt. under 25 g.			Wt. 25–30 g.			Wt. 30–35 g.			Wt. 35 g. and over		
	Rats with acrodynia		Rats without acrodynia	Rats with acrodynia		Rats without acrodynia	Rats with acrodynia		Rats without acrodynia	Rats with acrodynia		Rats without acrodynia
	No.	%	No.	No.	%	No.	No.	%	No.	No.	%	No.
No egg white	60	69·8	26	73	67·0	36	62	70·5	26	37	82·2	8
10% egg white	53	91·4	5	67	83·8	13	37	90·2	4	10	83·3	2

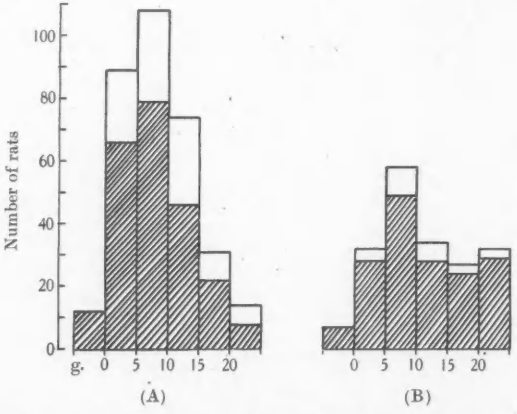

Fig. 1. Histograms showing relationship of incidence of acrodynia to gain in weight of rats (A) on the "no egg white diet" and (B) on the "10% egg white diet".

Discussion

The ideal diet for the production of acrodynia in rats would be one which supplied all the vitamins required by the rats except vitamin B_6. At present it is impossible to arrange such a diet because of the still fragmentary knowledge of the remaining factors of the vitamin B_2 complex.

In view of the data reported here, incorporation of egg white (up to 10%) in the basal diet originally devised [György, 1935, 1] for the production of rat acrodynia seems to improve results considerably. In the first place, the greater

gain in weight in the shorter time interval among the rats on the "10% egg white diet" indicates that this diet provides more completely the requirements of the rat than does the basal diet without egg white. This conclusion is also supported by the lower incidence of pneumonia seen in the rats on the egg white diet, as they are apparently better able to resist infection by reason of their better nutritional state.

With regard to the higher incidence and shorter time required to produce acrodynia in rats on the egg white diet, one is tempted to refer to the "concurrence of vitamin deficiency diseases" [György *et al.* 1937; György & Goldblatt, 1939]. This phenomenon is based on the observation that when an animal is placed on a multiple deficiency diet one of these deficiencies assumes the dominant role, relegating the other deficiencies to more latent positions. This has been the case with the original experimental diet used for the production of the vitamin B_6 deficiency disease. Rat acrodynia assumes the dominant role, suppressing the manifestation of the other deficiencies which are known to coexist. When a purified vitamin B_6 preparation or pure vitamin B_6 is given to a rat suffering from acrodynia, the acrodynia is cured and the other deficiencies may then become manifest. This is what occurs when panmyelophthisis develops in rats [György *et al.* 1937]. It was first observed in animals cured of acrodynia by the administration of relatively pure vitamin B_6 preparations. Occasionally, however, in the group of rats kept on the original basal diet without egg white here reported, acrodynia seemed to disappear spontaneously, without specific treatment, and the symptoms of panmyelophthisis became evident and assumed the dominant role over the acrodynia for reasons as yet obscure. The absence of these symptoms in the rats on the egg white diet tends to indicate that the function of the egg white is to supply some of the essential food factors, the addition of which seems to favour even more the suppression of panmyelophthisis. Inasmuch as the egg white diet does not definitely prevent the occurrence of panmyelophthisis in rats that have been cured of acrodynia by the addition to the diet of vitamin B_6, the conclusion is warranted that egg white does not supply the specific maturation factor for prevention of panmyelophthisis.

It may here be noted that the connexion of nicotinic acid with panmyelophthisis assumed on the basis of preliminary experiments [György, 1937–8] has not been borne out by subsequent observations. As stated by one of us (P. G.) in a report given at the Symposium on the Vitamin B Complex at the Annual Meeting of the American Institute of Nutrition in Baltimore, Maryland, in 1938, nicotinic acid was devoid of any curative effect on panmyelophthisis, and even the prophylactic effect, at first suggested [György, 1937–8], was lacking, or at least uncertain, in later experiments.

II. *Vitamin B_6 and cutaneous lesions in rats*

In pursuing the isolation of vitamin B_6, the assumption has been followed that the cutaneous manifestations which occur in rats kept on a diet devoid of the vitamin B_2 complex with the exception of riboflavin are due to lack of vitamin B_6. On the other hand, the lack of parallelism between growth-promoting activity and prevention of dermatitis [György, 1935, 1, 2] appeared to be based on the multiple deficiency represented by the diet employed for the production of rat acrodynia.

Later, Lepkovsky *et al.* [1936] conclusively showed that rats need another factor in the diet, in addition to vitamin B_1, riboflavin and vitamin B_6, to supply the requirements for normal growth and development when they are

fed a diet deficient in the vitamin B complex. This additional factor was thought to be identical with the so-called chicken pellagra factor or the filtrate factor [Elvehjem & Koehn, 1935]. Addition of vitamin B_6 (called also Factor 1 [Lepkovsky *et al.* 1936]) alone did not permit growth in rats, and "some of the rats developed swollen eyelids which tended to stick together. The eyes were watery and the nose was inflamed". Addition only of the filtrate factor (called also Factor 2) resulted in slow growth and in development of the specific acrodynia-like condition of the skin. Addition of both Factor 1 and Factor 2 assured normal growth and development. In the experiments of Lepkovsky *et al.* [1936] the separation of Factor 1 from Factor 2 was achieved by adsorption of the former on fuller's earth and subsequent elution by $Ba(OH)_2$, the procedure first recommended in the purification of vitamin B_6 by Birch & György [1936]. Factor 2 remained in the filtrate. Both these preparations represented rather crude concentrates with no appreciable degree of purification.

It is of interest to note that in rats no cutaneous lesions other than sore nose and sticky eyes, and in some instances loss of hair around the eyes ("spectacled eyes" [Oleson *et al.* 1939, 1]), have been attributed to lack of the filtrate factor, whereas the supposedly equivalent deficiency in chicks is characterized by extensive dermatitis. In view of these observations and of the fact that the cutaneous manifestations in rats fed on a diet deficient in riboflavin are less conspicuous, the designation *adermin* has been proposed by Kuhn & Wendt [1938] for vitamin B_6.

After the combined effects of Factor 1 and Factor 2 on the growth of rats were known, it became possible to devise experimental conditions for bioassays in which the growth-promoting activity of Factor 1 (vitamin B_6) could be utilized as the criterion. In their studies on vitamin B_6, Edgar *et al.* [1938] have relied mainly on this method. One should not overlook, however, the distinctly unspecific nature of the reaction of growth, and therefore the possibility that the promotion of growth, especially when crude concentrates are used, might result from the action of more than one constituent.

Working on similar lines to Edgar *et al.* [1938], Lepkovsky [1938, 1] and Kuhn & Wendt [1938] have also supplemented the basal diet containing vitamin B_1 and riboflavin with a crude concentrate of the filtrate factor, but, by giving equal significance to the curative effect on the acrodynia-like lesions in the skin, they have not relied solely on the growth-promoting activity of the vitamin B_6 concentrates tested.

In experiments on the isolation of vitamin B_6 we have repeatedly tried this procedure, except that the concentrates of the filtrate factor were added not at the beginning of the experiment but only after the first symptoms of acrodynia had become evident. We used several different preparations of the filtrate factor, some of which were kindly put at our disposal by Dr Lepkovsky and Dr Jukes, while others were prepared according to the methods described by Lepkovsky [1938, 1] and by Edgar *et al.* [1938]. A definite improvement in the cutaneous condition was almost always observed, together with some promotion of growth, even before any vitamin B_6 was added. In view of these results we suspected the presence of small, not completely removed amounts of vitamin B_6 in these crude concentrates of the filtrate factor. We therefore discontinued the use of these preparations as supplements and returned to the original basal diet, although it is admittedly incomplete in several respects, without or with 10% dried egg white and with only vitamin B_1 and riboflavin to represent the vitamin B complex.

Isolation and chemical characteristics of vitamin B_6

With this method of bioassay vitamin B_6 was isolated [György, 1938, 1] from yeast, independently of and at about the same time as Lepkovsky [1938, 1], Keresztesy & Stevens [1938] and Kuhn & Wendt [1938]. In the preliminary report of György [1938, 1] the various steps in the analysis which led to the pure crystalline product were described. They were the same, in the main, as the procedure used independently by Lepkovsky [1938, 2].

Whereas the pure base was found to be very soluble even in an alcohol-ether mixture containing 90 vol. % of ether, the vitamin B_6 chloride readily crystallized out from a water-acetone solution acidulated with HCl or from alcohol-dioxane, in a similar way to vitamin B_1. The chemical properties of this natural vitamin B_6 were in complete accord with those described by Keresztesy & Stevens [1938] and by Kuhn & Wendt [1938]. Sublimation (molecular distillation) was successfully employed for final purification. The melting point of a preparation thus obtained was found to be 203–204° (corr.) and was accompanied by the appearance of a brown discoloration and by decomposition, again in complete accord with the data given by Keresztesy & Stevens [1938] and by Kuhn & Wendt [1938].

The chemical and biological properties of this natural vitamin B_6 have been compared with samples of vitamin B_6 received from Merck & Co. Inc. through the courtesy of Dr Molitor and Dr Keresztesy, and with samples received from Prof. Kuhn. All three preparations were found to be identical.

Biological effect of vitamin B_6 on rats

Of particular interest was the biological effect of these crystalline vitamin B_6 preparations when they were administered to rats under the experimental conditions chosen, namely, as a supplement to the "no egg white diet" or to the "10 % egg white diet", both of which contained 20 μg. of aneurin and 20 μg. of riboflavin but lacked Factor 2 or any other member of the vitamin B_2 complex.

Therapeutic tests. In rats suffering from acrodynia, daily addition of 10 μg. of vitamin B_6 to the diet, and in many instances even 5 μg., brought about improvement of the specific dermatitis; in many rats it led to complete cure. A few rats, however, proved refractory and did not respond definitely to treatment with vitamin B_6. In general, promotion of growth was irregular and, as a rule, either slight and very transitory or lacking completely. Only exceptionally was the upturn of the weight curve well pronounced and sustained for several weeks. Weight curves of some of the rats are given in Fig. 2.

During the further course of the therapeutic experiments with the same rats the weight curves invariably became flat and even declined, a result which proves beyond doubt that despite the addition of vitamin B_6 the diet remained incomplete. Later, some of these rats died from different specific deficiency diseases, such as panmyelophthisis, adrenal haemorrhage with concomitant necrosis, and hepatic injury. In a large percentage of the rats that did not die, cutaneous manifestations persisted or developed anew after the specific acrodynia had been relieved or cured by treatment with pure natural vitamin B_6. As previously stated [György, 1938, 1], complete cure of rat acrodynia, with its effects of stunted growth and primary and secondary cutaneous manifestations, can be achieved only by the administration of a combination of vitamin B_6 and a second, unknown factor (or factors), which is (or are) present in the so-called filtrate factor.

As soon as synthetic vitamin B_6 became available,[1] it was possible to extend the foregoing observations by making tests on a larger scale and to confirm them in every detail. The use of very high doses (up to 100 μg. of synthetic vitamin B_6 daily) did not lead to materially better results than those previously obtained with daily doses up to 15 μg. of natural vitamin B_6.

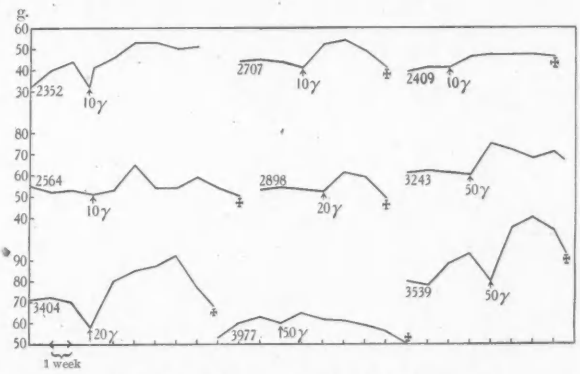

Fig. 2. Weight curves of 9 rats kept on the experimental diets, with or without egg white, showing effect of administration of vitamin B_6 (initial dose indicated at arrow). Cutaneous lesions of Type I developed in rats 2352, 2898 and 3977, of Type II in rats 2707, 2564, 3243, 3404 and 3539 and of Type III in rat 2409.

Prophylactic tests. Synthetic vitamin B_6 has been tested not only in therapeutic but also in prophylactic experiments. A large series of rats just weaned were put on the basal ration supplemented daily with 20 μg. of aneurin, 20 μg. of riboflavin and with 10, 20, 50 or 100 μg. of vitamin B_6. Additional supplements of 2 mg. of choline were administered to smaller groups of rats, either from the beginning of the experiment or after the signs of acrodynia had become apparent and the treatment with vitamin B_6 had been initiated.

In the prophylactic experiments the "no egg white diet" was mainly used as the basal ration, whereas the therapeutic effect of vitamin B_6 was tested in rats kept either on the "no egg white diet" or on the "10% egg white diet".

Later, in experiments with another group of rats, 500 mg. of choline were added per kg. of the diet. The addition of choline was considered necessary after severe renal injury had been observed in a relatively large number of young rats in the prophylactic experiments. This kidney lesion, details of which, together with a discussion of choline as a member of the vitamin B_2 complex, are given in a separate report [György & Goldblatt, 1940], is characterized by symmetrical haemorrhagic necrosis of the kidney cortex, of the type described by Griffith & Wade [1939] and Sure [1940] in choline deficiency, and by Hartwell [1928], Curtis & Newburgh [1927] and Cox *et al.* [1929] in cystine intoxication. It should be emphasized that the basal casein diet supplemented with vitamin B_1, riboflavin and vitamin B_6 is more liable to lead to lethal changes of this kind, visible to the naked eye at autopsy, than is the same diet without vitamin B_6. Addition of 2 mg. of choline daily or of 500 mg. of choline per kg. of the diet has invariably proved effective in the prevention of necrosis of the kidney cortex.

[1] Synthetic vitamin B_6 was generously put at our disposal by Merck and Co. Inc., Rahway, New Jersey.

These haemorrhagic renal lesions were observed only in very young rats, and therefore were absent from the older animals necessarily used in the therapeutic experiments.

In a few young rats of the choline-free group, flaccid paralysis of the hind legs was seen, which might be attributed also to choline deficiency, as recently described by Sure [1940].

A total of 507 rats was used in the therapeutic and prophylactic bioassays of vitamin B_6 (natural and synthetic). This number was distributed as follows:

(1)	On the "no egg white diet":	
	(*a*) Therapeutic tests (5–10 μg. of vitamin B_6) with or without addition of choline	143 rats
	(*b*) Prophylactic tests (10–100 μg. of vitamin B_6) with or without addition of choline	253 rats
(2)	On the "10% egg white diet": Therapeutic tests mainly	111 rats

In this large group of animals the preliminary observations [György, 1938, 1] were fully confirmed. Addition of vitamin B_6 to the basal diet supplemented daily with 10 μg. of aneurin and 20 μg. of riboflavin was unable to assure permanent restoration of growth with lasting cure or prevention of cutaneous lesions in the rats which, in the therapeutic experiments, failed to react appreciably or which showed, as a rule, only transitory improvement.

Corresponding weight curves of a few of the rats are given in Fig. 2.

The animals in which cure or prevention of cutaneous lesions could be considered complete or almost complete succumbed during the further course of the experiments to secondary diseases, such as adrenal haemorrhage, with or without panmyelophthisis, hepatic injury, and, of course, to unspecific secondary infections, chiefly pneumonia.

In a fairly large group of these rats no cause of death could be determined, and only complete failure to gain weight gave any indication of the presence of a basic metabolic disturbance, probably of the character of a deficiency disease.

Cutaneous lesions in rats treated with vitamin B_6

In the majority of rats treated with vitamin B_6 lesions of the skin became apparent both in the therapeutic and prophylactic experiments. Three types could be distinguished [György & Eckardt, 1939]; they were different from the condition seen in specific acrodynia in rats, known as vitamin B_6 deficiency.

Type I. The hair over the abdomen is matted together, appears damp and is sticky to the touch. There is loss of hair around the chin, nose and eyes and over the forehead between the ears. The sides of the cheeks and also of the abdomen are often covered with scales which are generally brown in colour. These scales may sometimes become quite thick. In advanced cases, the lids of the eyes seem to be inflamed and are at times kept closed, while the brown scaliness may extend over the sides of the forehead. On account of irritation, the animal often rubs his forehead and cheeks with his forepaws and thus probably transfers a coating of the brown scaly matter to the paws. There is emaciation and inanition.

Type II. After complete cure or improvement of the acrodynia, new lesions start around the mouth, the groin and the axillae, in the form of inflammation and production of scales. Later, alopecia follows and extends to the neck and over the back. Hyperaemia may be seen also on the ears and the forepaws often

become excoriated. In several animals generalized scaliness (exfoliative dermatitis) has been observed.

Type III. This form seems to be identical with the condition described by Lepkovsky *et al.* [1936] in rats that received a rather crude concentrate of vitamin B_6 (Factor 1) lacking Factor 2, or with that seen in similar circumstances by Oleson *et al.* [1939, 1], which they called "spectacled eye". This type is characterized by watery eyes, which stick together, by loss of hair (abrasion) around the eyes ("spectacle" appearance) and by sore mouth. In our own animals, progressive keratitis with final complete destruction of the eyeballs (panophthalmitis) would sometimes develop. The changes in the cornea were, as a rule, different from those seen in riboflavin-deficient rats [Eckardt & Johnson, 1939], but occasionally they resembled them closely. In this connexion it should be pointed out that rats with this type of lesion are commonly infested with pediculi and may show the same fine, creamy, often almost dandruff-like generalized scaliness that has been described [György, 1934; 1938, 2] in rats suffering from riboflavin deficiency.[1] A further analysis of this condition will be given in a later report, to be published separately.

Types I–III are progressive lesions and have a fatal outcome unless treatment is instituted in time (Fig. 2).

Fur alterations in rats treated with vitamin B_6

Apart from cutaneous lesions, fur alterations also can be observed in rats kept on a basal diet deficient in the vitamin B complex and supplemented with aneurin, riboflavin, vitamin B_6 and choline. The fur of these rats loses its pigment ("greying" effect) in piebald or black animals and shows "rusting" in albino rats. The fur changes appear to be identical with those seen by other investigators [Bakke *et al.* 1930; György, 1935, 1; Morgan *et al.* 1938; Lunde & Kringstad, 1939; Oleson *et al.* 1939, 2; György *et al.* 1940] in rats kept under similar conditions. They were found mainly in connexion with cutaneous lesions of Type II and somewhat less frequently with those of Type III. Their absence in connexion with Type I lesions might be explained by the fact that the rats in this group usually died very soon after development of the specific symptoms of Type I.

Discussion

From the observations reported it is evident that the routine use of crystalline vitamin B_6 in a diet devoid of the vitamin B complex supplemented with aneurin and riboflavin has had only a transitory effect in simplifying the problems encountered in the systematic analysis of the vitamin B_2 complex.

In prophylactic experiments the addition of vitamin B_6 to a diet deficient in the vitamin B complex supplemented with aneurin and riboflavin and containing casein as the source of protein has shed more light on the importance of choline as a necessary constituent, a fact which hitherto has not been sufficiently emphasized.

In further prophylactic and therapeutic experiments it has become clear that the problem of rat acrodynia, specifically that of the cutaneous lesions observed in rats fed on a diet lacking the vitamin B complex supplemented with vitamin B_1 and riboflavin, is not alone a question of vitamin B_6 deficiency. The three different types of cutaneous lesions seen in rats after the incorporation of vitamin B_6 in the experimental diets should be regarded as visible signs that the diets lack some other unknown members of the vitamin B_2 complex. This observation, that

[1] It should be noted that "spectacled eye" has also been previously seen in rats suffering from lack of riboflavin [György, 1935, 1].

vitamin B_6 is only one of the factors which are influential in the cure or prevention of cutaneous manifestations in rats kept on the experimental diets heretofore used for the production of rat acrodynia, is well in accord with the results recently reported by Schneider *et al.* [1940].

Summary

I. Incorporation of dried heated egg white in the experimental diet used for the production of rat acrodynia increased the incidence of the disease and shortened the time of its appearance.

II. 1. In young rats kept on a casein diet supplemented with aneurin, riboflavin and vitamin B_6, necrosis of the kidney cortex occurred as a symptom of choline deficiency more often than in rats on the same diet not supplemented with vitamin B_6.

2. Rats with acrodynia react only transitorily to vitamin B_6. Cutaneous lesions, which can be classified into three different groups, and achromotrichia are regularly observed in the rats treated with vitamin B_6 without the addition of other members of the vitamin B_2 complex, apart from riboflavin and choline.

3. Attention is called to the severe lesions about the eyes (keratitis, panophthalmitis) observed in one group of these rats; in this group pediculosis was often observed.

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137. THE CHEMICAL NATURE OF ROYAL JELLY[1]

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Received 14 June 1940

Most chemical investigations of substances important in nutrition have dealt with foods used by mammals. The work to be reported is an attempt to determine the chemical nature of the components of royal jelly, the peculiar nutriment which is fed to queen bees by their subjects throughout life. Royal jelly is a thick, milky material which is generally believed to be a secretion of the pharyngeal glands of the nurse bees (young workers). For a proper appreciation of the nutritional significance of royal jelly the following facts about the natural history of bees should be recalled.

A colony of bees consists of one queen, several hundred drones or males and from 25,000 to 100,000 worker bees, sexually undeveloped females. This large number of workers are all the daughters of one queen. As the bees die off constantly the queen may, during the whole season, become the mother of a quarter of a million bees. At the height of the season, queens have been known to lay more than 2000 eggs (over their own weight in eggs) in one day. Such fecundity must be associated with a very active metabolism and the nature of the food which makes this possible is of interest. But of greater nutritional significance is the fact that the anatomical and physiological differentiation of the female larvae is dependent upon the nature of their diet. Two types of eggs are laid by the queen: (1) unfertilized eggs which give rise only to drones (male bees), and (2) fertile eggs which give rise to females, either workers (sexually immature females) or queens. By grafting young larvae, it has been proven that a nutritional difference determines whether a fertile egg is to become a worker or a highly specialized egg-laying organism, the queen. All female larvae are fed on royal jelly for the first 2–3 days after hatching and during this period their anatomical development is similar. Only the queen continues to receive this special diet. Any larva from a fertile egg, if given royal jelly throughout its larval period, develops sexually so that it becomes a perfect or true female bee, or what is called a queen; otherwise, the larva develops into a sexually immature worker. The queen is structurally much the same as the workers but with these important differences: the pollen-gathering apparatus remains undeveloped, the mouth parts and sting are modified, while the spermotheca and ovaries are highly developed.

Previous chemical work on the nature of royal jelly is very limited. The first three papers dealt only with proximate analyses, since the workers were mainly concerned with nutritional aspects of the problem. The early analyses carried out by Von Planta [1888] and more recently confirmed by Aeppler [1922] and

[1] In view of the fact that one of the authors (G. F. T.) is changing his post, it has been considered advisable to publish this preliminary report, although several of the fractions have not yet been investigated very thoroughly.

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Elser [1929] indicate a similarity in the food of the worker and the queen larvae during the first 2–3 days of larval development. The food of the larvae destined to become workers is changed on the third day to one much richer in carbohydrate material (honey and pollen) while the queen continues to be raised on royal jelly. Several months ago Melampy & Jones [1939] published the results of a careful study of the proximate chemical composition and vitamin content of royal jelly collected in Louisiana, U.S.A. The average values indicated 66% moisture, 0·82% ash, 12·34% protein, 5·46% total lipid, 12·49% total reducing substance (by the dinitrosalicylic acid method of Sumner) and 2·84% undetermined. Vitamin A could not be detected, vitamin B_1 was present (1–1·5 international units per g.), but little, if any vitamin C was found in the sample studied.

With the discovery of vitamin E, it was thought that possibly royal jelly owed its astonishing sex-differentiating property to the presence of large quantities of this vitamin. Hill & Burdett [1932] performed experiments to test this interesting hypothesis and claimed that if pregnant rats on vitamin E-deficient diets were fed 50 mg. of royal jelly per day it would prevent resorption of the embryos. In 1936, Melampy & Mason subjected the above work to critical examination and found several possible sources of error in both the technique and conclusions of Hill & Burdett. Melampy & Mason repeated the experiments under better controlled conditions and found that even as much as 1 g. of royal jelly fed daily throughout the period of gestation does not supply sufficient vitamin E to permit the completion of a normal pregnancy in E-deficient female rats. This finding has been thrice confirmed [Schoorl, 1936; Evans, *et al.* 1937; Haydak & Palmer, 1938]. It would appear, therefore, that some nutritional factor other than vitamin E is responsible for the production and maintenance of queen honey bees.

Quite recently Heyl [1939] has described observations which suggest that a gonadotropic hormone occurs in royal jelly. Dilute NaOH extracts or aqueous pyridine extracts of royal jelly, when injected subcutaneously for 5 days into 21-day-old female rats, caused an increase in weight and in follicular activity of the ovaries. This observation may be related to the finding in this laboratory, that the ether-soluble fraction of royal jelly, when added to the diet of fruit-flies (*Drosophila*), caused a marked increase in the rate of reaching sexual maturity and also increased the number of eggs laid per day (by about 60%). These experiments will be reported in detail elsewhere.

Experimental

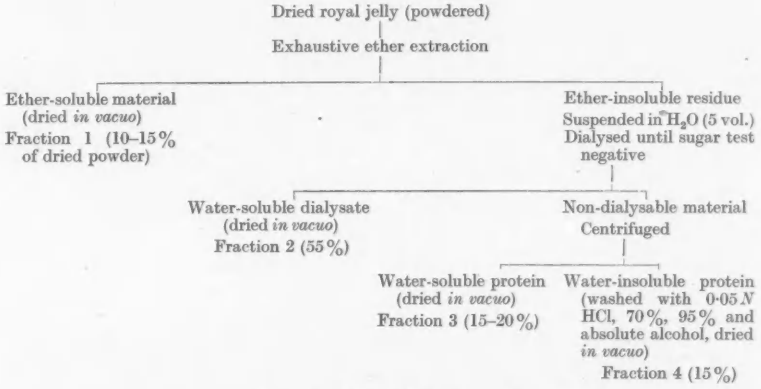
The collection of sufficient royal jelly for investigation is a matter of great difficulty since normally one finds only a very few queen cells per hive and the amount of material per cell is very small. The royal jelly was collected from the bottom of queen cells containing larvae 2–3 days old. The queen cell was cut from the frame, the larva removed and the jelly squeezed out.

The small individual samples of royal jelly were pooled and kept in a refrigerator at about 0° until all the available material had been collected. This amounted to about 100 g., over 20 g. of which was used in feeding experiments to be described elsewhere. Royal jelly from different parts of Ontario was kept separate. Each sample was weighed and dried over $CaCl_2$. Towards the end of the drying period the material was powdered, screened and returned to the desiccator. It was finally dried over P_2O_5 *in vacuo*, at room temperature, until it reached constant weight. This process took approximately 6 weeks. The various samples of royal jelly, as received, were found to vary in moisture content from 45 to 65%.

Preliminary fractionation

The dried material was extracted exhaustively with ether in a Soxhlet extractor and the residue, suspended in water, was dialysed. Four main fractions were thus obtained as shown in Table 1.

Table 1


Dried royal jelly (powdered)
Exhaustive ether extraction
Ether-soluble material (dried *in vacuo*) Fraction 1 (10–15% of dried powder)
Ether-insoluble residue Suspended in H_2O (5 vol.) Dialysed until sugar test negative
Water-soluble dialysate (dried *in vacuo*) Fraction 2 (55%)
Non-dialysable material Centrifuged
Water-soluble protein (dried *in vacuo*) Fraction 3 (15–20%)
Water-insoluble protein (washed with 0·05 *N* HCl, 70%, 95% and absolute alcohol, dried *in vacuo*) Fraction 4 (15%)

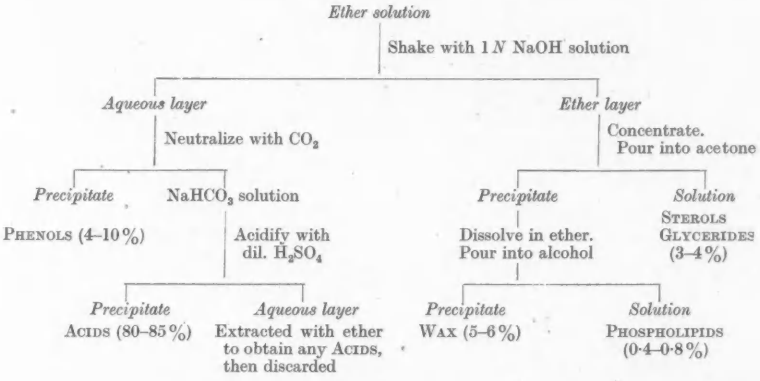
Examination of the fractions

Fraction 1. The ether-soluble material was a creamy coloured semi-crystalline solid of waxy consistency with a faint flowery to spicy odour. It contained 0·07% phosphorus and 0·3% nitrogen. The fraction was redissolved in ether and shaken with *N* NaOH to remove acids and phenols. After several such treatments about 90% of the ether-soluble material went into the alkaline aqueous layer. The complete fractionation is shown in Table 2.

After the treatment with 1 *N* NaOH, the ether layer ("lipid" fraction) was dried with anhydrous Na_2SO_4, evaporated to dryness and weighed. The residue was redissolved in the smallest possible amount of ether and poured into several vol. of acetone. A small precipitate (about two-thirds of the "lipid" fraction or 6% of fraction 1) was formed. This acetone precipitate should contain phosphatides and waxes. After being dried and weighed, it was taken up in ether and poured into alcohol. The resulting precipitate, which contained neither N nor P, melted sharply at 69° and on reprecipitation the M.P. remained unchanged. It accounted for about 5% of fraction 1 and was apparently beeswax. Purified beeswax from different sources melts at temperatures between 65 and 70° and is precipitated by alcohol or acetone from ether solution in exactly the same manner. The minute amount of alcohol-soluble, acetone-insoluble material (about 0·4% of fraction 1) is apparently phospholipin but it has not been examined.

The ether-acetone solution was evaporated to dryness and weighed. This "fat" fraction accounts for about 3–4% of fraction 1. One portion of the small residue was dissolved in chloroform and tested for cholesterol. The Liebermann-Burchard reaction was strongly positive. Another portion was used for a micro-determination of the saponification number; the values found were 116 and 118. These low figures may indicate sterol esters or glycerides mixed with free sterols.

Table 2. *Fractionation of ether-soluble material extracted from dried royal jelly*



Owing to the minute amount of material available, this fraction was not further characterized.

The alkaline extract obtained from the original ether solution of fraction 1 was treated with CO_2 to set free the phenols. A small amorphous precipitate with the characteristic flowery to spicy odour (reminiscent of cloves) was obtained. The aqueous solution was extracted with ether to remove completely any phenols. Phenols accounted for 4 and 10%, respectively, of fraction 1 in two different samples of royal jelly which were worked up separately to this stage. The crude phenol melted at 65°; when recrystallized from dilute alcohol and then from aqueous acetone it melted at 69°. An attempt to identify it as the aryloxyacetic acid derivative gave no crystalline product.

The alkaline aqueous extract (freed of phenols) was acidified with H_2SO_4 and extracted with ether. The ether solution was dried over anhydrous sodium sulphate and evaporated. An amorphous residue with low indistinct melting point resulted. It was taken up in hot alcohol, and water was added to produce turbidity. The solution was warmed to clear it; on cooling slowly crystals separated. They were not well formed, were of a soft, waxy consistency and melted over a wide range (45–56°). When recrystallized from aqueous acetone, long prisms and poorly formed plates melting at 45–56° were obtained. Titration in dilute alcohol with 0·1 *N* NaOH gave a sharp end-point corresponding to an equivalent weight of 178. Fractional crystallization from aqueous acetone and from aqueous alcohol failed to resolve this acid fraction into materials of sharper melting point or of different equivalent weight. It appears to soften about 46 to 48° and to melt at 58°, but not sharply. The acid is probably not yet pure; further attempts to fractionate the material are being made. In the meantime, the following data may be recorded. A 10% solution in absolute alcohol is optically inactive. The free acid is insoluble in cold water and only slightly soluble in hot water; it is soluble in alcohol, ether, acetone, chloroform and benzene, but it is insoluble in light petroleum. The Na and Ba salts are soluble in water, the Pb and Cu salts are insoluble. It does not contain N, S or P.

A micro-analysis (Schoeller) gave C = 64·23, 64·12% and H = 9·48, 9·63% with a trace (0·19%) of ash residue. These figures agree fairly well with the

formula $C_{10}H_{18}O_3$; calculated C = 64·45 %, H = 9·68 %, mol. wt. = 186. The acid (or mixture) does not absorb Br in chloroform solution and is therefore not unsaturated. It does not lose weight when kept slightly above its melting point for several hours *in vacuo* over P_2O_5 and is apparently not an anhydride-acid or lactone-acid mixture, since titrations at 0 and 100° give the same values. Further work on this acid will be done as soon as more material can be obtained.

Fraction 2, the water-soluble, dialysable material, was evaporated *in vacuo*. The resulting syrupy solution was transferred to a wide dish and put in a vacuum desiccator over P_2O_5. The drying agent was renewed frequently for several weeks and the sticky residue was kept broken up to hasten drying. When brought to constant weight the toffee-like residue accounted for about 55 % of the dried royal jelly. It contained 1·2 % N, 0·37 % inorganic P and 0·82 % total P. Upon ignition, 227 mg. left a residue of 7·8 mg., corresponding to 3·4 % ash. It gave negative tests for chloride and sulphate. The test for Ca was positive.

A portion of fraction 2 was dissolved in water to give a 20 % solution, from which 5 and 1 % solutions were prepared. The 1 % solution was acid in reaction (*p*H 3·6). Sufficient of the 5 % solution to contain exactly 100 mg. of the residue required 5·2 ml. 0·01 *N* NaOH to neutralize it to phenolphthalein. Another preparation of this fraction obtained a year later required 6·4 ml. 0·01 *N* $Ba(OH)_2$ at 0° and 7·3 ml. at 50°. Such neutralized solutions reduced Fehling's solution strongly; even in the cold there was a slight reduction in 7–8 min. The behaviour suggested the presence of uronic acids as well as sugars. Tollen's naphthoresorcinol test, with benzene extraction [Neuberg & Saneyoshi, 1911] was negative when applied directly, but since glucose is known to interfere [Brule, *et al.* 1925] the solution (containing 100 mg. fraction 2) was treated first with 0·3 ml. 20 % lead acetate and then with 2 ml. basic lead acetate to precipitate any uronic acids, thus separating them from sugars. The precipitates were separately washed twice with water, treated with 1:1 HCl and 0·5 ml. 1 % alcoholic naphthoresorcinol solution on a boiling water bath and the cooled mixtures were extracted with benzene. The neutral lead precipitate gave rise to a green-red fluorescent coloration and a dark turbidity in the aqueous layer; the benzene extract was a pale reddish-brown by transmitted light, faintly bluish-purple by reflected light. While this was not a typical positive test for uronic acids, the behaviour seemed to be suggestive. Fructose, if present, would cause a reddish-brown colour in the aqueous solution and a yellow-brown coloration of the benzene extract, but interference is not due to fructose, since in known solutions it was readily separated by the lead procedure. The basic lead precipitate gave an even less satisfactory test. Even when 5 mg. glucurone were added to 100 mg. of the fraction, the test (after precipitation with lead) was weaker and more reddish-brown in colour than the blue-violet coloration obtained from 5 mg. glucurone in simple solution treated similarly. If a uronic acid is present, interfering substances obscure the test. Attempts to improve the colour by fractional precipitations with lead were unsuccessful. Ascorbic acid was found to behave somewhat similarly to the unknown in royal jelly; with naphthoresorcinol it gave a pale reddish-brown colour in the benzene extract. However, the aqueous solution of fraction 2 failed to reduce $AgNO_3$ in acid solution, although ascorbic acid in simple solution or added to fraction 2 did so even at room temperature. Identification of the material responsible for the reaction observed awaits further investigation.

Fraction 2 gave positive Seliwanoff and Ihl-Pechmann tests for ketoses. On one sample a Willstätter-Schudel titration indicated that 40 % of fraction 2 was aldose; a sample obtained later contained less aldose, about 20 % of the

fraction. Total reducing sugars were determined on the first sample by copper reduction according to the A.O.A.C. methods before and after fermentation with washed yeast (*S. cereviseae*), indicating 49 % and less than 1 % respectively.

A differential fermentation was carried out on the second sample of fraction 2 through the kindness of Dr T. F. Nicholson. This sample contained 53 % of total reducing sugars, calculated as glucose, determined by a modified Shaffer-Somogyi copper reagent [Harding & Downs, 1933]. By the use of a series of micro-organisms with specific fermentative or "removal" ability [Harding & Nicholson, 1933; Nicholson, 1936] this particular sample of fraction 2 was found to contain 52 % of its reducing material as fructose, 42 % as glucose and 6 % as non-fermentable matter. No other sugar was recognized by this procedure. There was no significant increase in reduction after hydrolysis with cold 2 *N* H_2SO_4 for 5 hr. or hot 0·5 *N* H_2SO_4 for 30 min.

An attempt to isolate the acid material was made as follows: Several grams of the original residue (fraction 2) were taken up in about 20 ml. water. A small insoluble portion which was filtered off was found to be soluble in alcohol; it is still unidentified. The water solution was treated with an excess of $BaCO_3$ to form Ba salts and evaporated on a water bath to a thick syrup. This was rubbed up four times with 95 % alcohol to remove sugars. The alcohol-insoluble Ba salts were dissolved in water and the excess $BaCO_3$ filtered off. The Ba salts in solution (volume reduced to 4 ml.) were precipitated with 6 vol. of alcohol. The precipitated Ba salt was taken up in water and precipitation was repeated twice more with 6 vol. of alcohol. In each case, about one-fourth of the bulk of the precipitate failed to redissolve in water. The salt was dissolved in water, Ba removed exactly with dilute H_2SO_4 and the solution was evaporated to a small volume, *in vacuo*, but would not crystallize on long standing. It was finally brought to constant weight over P_2O_5 *in vacuo*. An attempt to estimate the approximate equivalent weight of this material gave a value of 346. If this figure be accepted, the original titration indicates that the acid accounts for about 20 % of fraction 2. We have not as yet been able to isolate a pure compound in crystalline form or any crystalline derivative of this acid.

The alcoholic supernatant, freed of Ba and concentrated to remove alcohol, was made *N* with respect to H_2SO_4. It gave a precipitate with phosphotungstic acid, but not all of the nitrogen in the solution was precipitated. The nitrogenous compounds in fraction 2 have not yet been definitely identified.

Fraction 3, the water-soluble protein, was dried over P_2O_5 *in vacuo* to constant weight and found to contain 0·5 % ash, 14·9 % N, 0·3 % P and 0·89 % S. Qualitative tests for tryptophan, tyrosine, cystine and arginine were found to be positive; the test for histidine was faintly positive. The protein (1·07 g.) was hydrolysed with 8*N* HCl for 12 hr., at which time the amino-N was found to be at a maximum (80 % of the total nitrogen). After removing as much HCl as possible by repeated evaporation *in vacuo*, the mixture was diluted with H_2O and the black insoluble melanin filtered out. This was found to make up 1·7 % of the original weight and to contain 0·5 % of the total nitrogen.

The amino-acid solution was brought to a definite volume. One portion was used to determine a conventional N-distribution and smaller aliquots were removed for special tests.

When more of this fraction became available, a slightly larger portion (3·8 g.) of the protein was hydrolysed in the same manner. After removal of excess HCl and of melanin, the solution was made alkaline with baryta and concentrated *in vacuo* (below 40°) to a small volume. The solution of the Ba salts of the amino-acids was treated with 4 vol. of alcohol to precipitate the dicarboxylic acid

Table 3. *Nitrogen distribution of water-soluble protein*

(15·08% N, on moisture and ash-free basis)

Melanin-N	<1% of total N
Ammonia-N	13
Dicarboxylic acid-N	10
Basic N	21
Non-amino-N	20
Mono-amino-N	35

fraction. This fraction was taken up in the smallest possible volume of water and reprecipitated once with 3 vol. of alcohol and finally with 2 vol. of alcohol. The dicarboxylic acid fraction thus obtained contained nearly 20% of the total N; however, both tyrosine and arginine were still present in small amount and some glycine, although not definitely identified, was probably also present. No glutamic acid could be isolated either before or after removal of aspartic acid. The latter was isolated as the copper salt in a yield corresponding to 9·9% of aspartic acid in the original protein. The combined filtrates, after removal of alcohol, were freed of Ba with H_2SO_4, of Cl with Ag_2SO_4 and of Ag with H_2S.

The filtrate from this procedure was made *N* with respect to H_2SO_4 and treated with phosphotungstic acid to precipitate the basic amino-acids. The filtrate from this was freed of all inorganic ions and concentrated *in vacuo*, upon which 17 mg. tyrosine crystallized out; the remaining amino-acids were converted into copper salts.

The phosphotungstate precipitate was dissolved in aqueous acetone and freed of inorganic ions with baryta. Arginine was precipitated as flavianate (M.P. 249°), corresponding to 10% of the original protein. Neither histidine nor lysine could be obtained as crystalline derivatives, so no quantitative data concerning them are yet available, but they cannot be present in more than traces since the arginine isolated accounts for practically all of the basic N.

The copper salts have not yet been adequately examined.

Fraction 4, the water-insoluble protein, dissolves only in alkalis, e.g. 0·5% Na_2CO_3. It contains 13·4% N and 0·16% P. It contains S, but no Pb-blackening S. Tests for tyrosine, tryptophan and arginine are positive. It has yet to be investigated.

Summary

Royal jelly has been separated into four main fractions: (1) ether-soluble, (2) water-soluble and dialysable, (3) water-soluble, non-dialysable and (4) water-insoluble.

Fraction 1 appears to consist principally of an organic acid (or mixture of acids), M.P. 58°, equivalent weight, 178. Small amounts of phenolic material and smaller amounts of beeswax, sterol, phospholipin and a saponifiable substance also occur in this fraction.

Fraction 2, the largest fraction, is about 50% sugars; glucose and fructose account for most of the reducing material. An unidentified acid, inorganic salts and nitrogen compounds are also present.

Fraction 3 is protein in nature. Its N distribution has been determined; aspartic acid, arginine and tyrosine were isolated; colour tests for tryptophan, cystine and histidine are positive.

Fraction 4 is a protein, soluble only in alkalis. It gives positive colour tests for tyrosine, tryptophan and arginine.

There is some evidence that fraction 1 contains the physiologically active material responsible for sexual development of the queen bee.

The authors wish to thank the Dominion Apiarist, Mr C. B. Gooderham, and also Dr E. J. Dyce and Mr W. G. Le Maistre, Department of Apiculture, Ontario Agricultural College, for help in collecting the royal jelly.

They are also grateful to Sir Frederick Banting for encouragement in initiating the study and for his continued interest.

This work was financed by a grant made by the Banting Research Foundation to the Department of Medical Research.

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138. CARBONIC ANHYDRASE. PURIFICATION AND NATURE OF THE ENZYME

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(*Received 3 June 1940*)

I. Introduction

Under the name of carbonic anhydrase Meldrum & Roughton [1933][2] described a new enzyme present in red blood corpuscles which catalyses both phases of the reversible reaction: $H_2CO_3 \rightleftharpoons CO_2 + H_2O$. They purified this enzyme from mammalian erythrocytes and gave a detailed account of its properties and the kinetics of the reactions which it catalyses. The greatly purified enzyme preparation obtained by them had a very high catalytic activity, was a colourless substance free from haematin and from other known enzymes, stable within *p*H 3–12, thermolabile, and very sensitive to KCN, H_2S, NaN_3 and to several heavy metals. The amount of the purified enzyme obtained by these workers was, however, very small and hardly sufficient to recognize it with certainty as a protein compound. The true nature of this enzyme and especially of its active group remained unknown until very recently.

While studying the metallo-protein compounds present in the red blood corpuscles we have found that the fractions of our preparations left after complete removal of haemocuprein [Mann & Keilin, 1938] had a very high content of Zn and a high carbonic anhydrase activity. By adapting our methods of purification to preparations on a larger scale, taking special care to increase the yield of the Zn fraction, we have obtained from the red blood corpuscles of the ox a highly active preparation of carbonic anhydrase as a colourless protein containing 0·31–0·34 % Zn. These results, together with some other considerations, have led us to the conclusion, put forward in a preliminary communication [Keilin & Mann, 1939], that the carbonic anhydrase is a Zn-protein compound.

In this paper we propose to give a detailed account of the methods of purification of this enzyme and to bring forward fresh evidence of its Zn-protein nature.

II. Methods of identification and estimation of Zn

A. *Qualitative tests*

In purified preparations of carbonic anhydrase Zn was identified by the following three tests:

(1) *As Zn quinaldinate*, $(C_{10}H_6NO_2)_2Zn, H_2O$. 25 mg. pure carbonic anhydrase in 5 ml. water are treated in a small centrifuge tube with 2 ml. 20 % trichloroacetic acid; after 15 min. standing the precipitate is centrifuged off and washed with dilute trichloroacetic acid. The combined clear, protein-free extracts are adjusted with ammonia to *p*H 6 and treated with one drop of concentrated acetic acid and

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[2] An interesting historical account of the work by other investigators which led Meldrum & Roughton to the discovery of this enzyme can be found in their paper [1933] and in the two excellent reviews of the whole subject by Roughton [1934, and especially 1935].

0·2 ml. 1 % solution of sodium quinaldinate. The crystallization of Zn quinaldinate begins almost immediately and is completed within a day.

(2) *As Zn mercury thiocyanate*, $Zn\,Hg(SCN)_4$ [Jamieson, 1918]. The organic matter of 120 mg. pure carbonic anhydrase is destroyed by heating with 2 ml. conc. H_2SO_4 in a Kjeldahl flask over a small flame. The water-clear solution is diluted to 12 ml. with water and treated with 2 ml. Jamieson's reagent (ammonium mercury thiocyanate). The crystallization of zinc mercury thiocyanate begins immediately and is completed within an hour.

(3) *As "Rinmann's green"*. The crystalline Zn mercury thiocyanate precipitate obtained as described above is centrifuged off and suspended in 0·3 ml. 10 % HNO_3; two drops of the mixture are deposited on a small strip of potassium cobalticyanide paper [Feigl, 1937] which is dried on a platinum plate and ashed over a gas flame. The green colour of the ash which appears immediately is due to the formation of mixed crystals of zinc oxide and cobaltous oxide.

B. *Quantitative estimations*

For quantitative estimation of Zn in blood and different fractions of carbonic anhydrase preparation two methods have been used:

(1) *Formation of Zn-diphenylthiocarbazone complex salt* or the red Zn-"dithizone" [Schwaibold *et al.* 1938]. Not less than 10 μg. and not more than 100 μg. Zn were used for a single determination. All glass containers must be carefully washed with sulphuric-dichromate mixture followed by HCl and rinsed in glass-distilled water. The reagents must be Zn-free, especially the sodium tartrate and dithizone. The solution of dithizone in CCl_4 is washed with dilute H_2SO_4 followed by several washings with glass-distilled water. The blank should not contain more than 2 μg. Zn. The samples for estimation are usually incinerated with H_2SO_4 in a Kjeldahl flask heated over a small flame. Samples containing a large amount of protein, as is the case with red blood corpuscles or very crude preparations, are evaporated to dryness and ashed for 5 hr. in an electric muffle.

Example of estimation: 2 ml. solution of carbonic anhydrase containing a known dry weight are incinerated with 1 ml. of pure Zn-free H_2SO_4. The colourless acid solution thus obtained is diluted with 50 ml. glass-distilled water and transferred quantitatively into a separating funnel. To make sure that the solution is free from copper, which combines with dithizone at acid *p*H, the solution is shaken with a few ml. of 0·006 % dithizone in CCl_4. The presence of Cu is revealed by the change in colour of dithizone from green to purple. If such change takes place successive portions of dithizone are added to the acid solution until the colour of dithizone remains unchanged. With a pure or highly purified sample of carbonic anhydrase the addition of dithizone at acid *p*H is unnecessary. The solution in the separating funnel is then treated with 3 ml. 20% sodium tartrate and rendered alkaline with ammonia, a 5 % solution of which is added slowly until a drop of the solution mixed on a spot-test plate with a drop of thymol blue gives a bluish colour. The solution is then shaken with a small amount of 0·006 % dithizone in CCl_4 which turns red or purplish red. The treatment is repeated several times with small portions of dithizone until the latter remains distinctly bluish-green in colour. The combined red Zn-dithizone fractions are diluted with CCl_4 to a definite amount, transferred into a clean separating funnel and washed several times with small portions of a freshly prepared 0·04 % solution of Na_2S which turns yellow and the washing is continued until the solution of sodium sulphide is left colourless. The red Zn-dithizone solution is then compared colorimetrically with a standard solution of Zn treated with dithizone in a

similar way. It is important to note that the Zn-dithizone solution should not be exposed to light more than necessary as it changes colour from red to brown.

(2) *Formation of potassium-Zn-ferrocyanide* estimated iodimetrically [Sahyun & Feldkamp, 1936]. This method of estimation requires, however, a much larger amount of Zn than that with dithizone and was only used with some of the fractions at the earlier stages of purification.

III. Estimation of catalytic activity of carbonic anhydrase

The activity of carbonic anhydrase in different preparations was estimated by two methods.

(1) *Manometric method* with the aid of the boat apparatus of Brinkman *et al.* [1933] based on the rate of evolution of CO_2 from sodium bicarbonate solution when mixed with phosphate buffer at *p*H 6·8.

In this method one side of the glass boat receives 2 ml. of phosphate solution prepared by mixing equal volumes of $M/5$ Na_2HPO_4 and $M/5$ KH_2PO_4, whilst in the other side are placed 2 ml. of $M/5$ $NaHCO_3$ dissolved in $M/50$ NaOH. In catalysed reaction a known amount of enzyme preparation is added to the side of the boat containing the phosphate solution. The experiments are carried out as described by Meldrum & Roughton [1933]. We have adopted also their definition of enzyme unit (E.U.), namely as the "amount of enzyme which, when dissolved in 4 ml. of the phosphate bicarbonate mixture used above, gives a value of $(R–Ro)/Ro$ equal to 1 at 15°" where Ro and R are respectively, the rates of reaction in the absence and in the presence of the catalyst.

The activity (A) of an enzyme preparation is expressed as the number of enzyme units (E.U.) per 1 mg. of dry weight of the preparation. The greater is this activity the smaller is the dry weight per enzyme unit. Thus, the enzyme preparation of which $A = 2000$ has $0{\cdot}5\mu$g. dry weight per enzyme unit which can be expressed as $0{\cdot}5\mu$g./E.U.

(2) *Colorimetric method* of Philpot & Philpot [1936] slightly modified. 10 ml. $0{\cdot}00263M$ solution of $NaHCO_3$ with a few drops of phenol red are put into a wide reaction tube kept at 0°. The current of CO_2 is bubbled through the solution and a few minutes later, while the current is still on, 2 ml. of $0{\cdot}3M$ Na_2CO_3 containing $0{\cdot}206M$ $NaHCO_3$ are added to the mixture. The reaction which is required to change the colour of phenol red from purplish red to pale yellow is carefully timed and is found to be from 65 to 75 sec. in uncatalysed reaction. The addition of carbonic anhydrase shortens this time and an enzyme unit (E.U.) by this method of estimation is defined as the amount of enzyme which is required to shorten the time of the reaction from 65–75 sec. to 25–30 sec. The enzyme unit determined in this way is about ten times larger than the unit of Meldrum & Roughton. The colorimetric method requires less manipulation and is much more rapid than the manometric method. It was found more convenient for quick estimations of enzyme activities and was therefore used as the main routine method for testing different fractions during the purification of the enzyme. The final values for the activities (A) of all the enzyme fractions described in this paper were estimated by Meldrum & Roughton's method.

IV. Material and general lines of purification

The highest concentration of carbonic anhydrase in nature is found in the red blood corpuscles [Meldrum & Roughton, 1933] and in the mucous membrane of the stomach [Davenport, 1939] of mammals.[1]

[1] The distribution of this enzyme in different animals, vertebrates and invertebrates was recently reviewed by Goor [1940].

The purification of the enzyme from erythrocytes of ox blood was carried out by two different methods which can be applied with the same success to the blood of sheep and with somewhat less success to blood of man and other mammals.

In both methods of purification the first step consists in treating the washed and plasmolysed red blood corpuscles with a mixture of alcohol and chloroform [according to Tsuchihashi, 1923] which denatures and precipitates the whole of the haemoglobin, leaving a clear yellow extract containing the enzyme. The similarity of these two methods does not go beyond this first step. The next step of purification in one method (I) consists in precipitation of the alcohol-chloroform extract, with lead acetate, elution of the precipitate with alkaline phosphate and further purification with $Ca_3(PO_4)_2$ and alumina Cγ. In the second method (II) the alcohol-chloroform extract is dialysed, precipitated with ammonium sulphate redissolved in H_2O and fractionated with ammonium sulphate and alumina Cγ.

The main advantages of method I consist in omitting the dialysis of a very large bulk of alcohol-chloroform extract and in securing a larger variety of enzyme fractions together with the fraction containing the haemocuprein. The defect of the method is in its comparatively smaller yield of the purified enzyme.

The advantages of method II are in its shortness and the greater yield of the final product. Its main defect is the necessity to dialyse a large amount of alcohol-chloroform extract.

The preparation of the enzyme from sources other than erythrocytes was carried out only from the mucous membrane of pig's stomach. The method used in this purification is, however, entirely different from the previous two methods applied to blood and will be described later on.

V. Purification of carbonic anhydrase from red blood corpuscles of the ox

A. *Method I*

(1) *Washing and plasmolysis of erythrocytes.* The red blood corpuscles of 7 l. of defibrinated ox blood are separated from serum and washed with an equal volume of 0·9% NaCl. The washed erythrocytes are plasmolysed with an almost equal amount of distilled water which brings the volume of the solution to 7 l. This solution contains 0·14 g. dry weight per ml. and 0·0005 ml. contains 1 E.U.; 70μg./E.U.; $A = \frac{1000}{70} = 14{\cdot}3$; 7 l. contain 14,000,000 E.U.; 0·0024% Zn. Zn present in this solution is not dialysable but can easily be liberated by trichloroacetic acid.

(2) *Extraction with alcohol and chloroform.* The solution of 7 l. of laked erythrocytes is cooled in ice and treated with ice-cold mixture of alcohol and chloroform [Tsuchihashi, 1923]. The best results are obtained by treating it in two or three successive lots in the following way: 2 l. of laked corpuscles are mixed with 1·8 l. 90% alcohol and 130 ml. chloroform. The mixture, which is made in a wide cylindrical jar, is rapidly stirred with a thick glass rod until the denatured haemoglobin sets into a large spongy mass which can be removed from the rest of the solution, pressed out and put aside for further washing. The fluid is mixed with kieselguhr and filtered on a large Büchner funnel (30 cm. diameter) through a filter paper covered with kieselguhr.

The residual cakes from the successive lots are collected, broken into small pieces, washed with about 2 l. of water and filtered in the same way.

The joint filtrates consist of 12 l. of clear yellowish fluid containing 2·5 mg. dry weight per ml.; 7·6μg./E.U.; $A = 133$; 3,947,000 E.U.; 0·027% Zn. The yield at this stage varies from 26 to 46%.

(3) *Precipitation with lead acetate.* 12 l. of cooled alcohol-chloroform solution are treated with saturated solution of lead acetate until no further precipitate is formed by the addition of a drop of this reagent to a few ml. of the filtrate. Usually, 25–35 ml. of saturated lead acetate per l. are required for this purpose. After 10–15 min. standing, the precipitate is centrifuged off, suspended in about 300 ml. of $M/5$ K_2HPO_4 and after 2–3 hr. standing again centrifuged. The brown fluid collected is dialysed for 24 hr. against distilled water, giving 750 ml. of brownish fluid.

(4) *Purification with $Ca_3(PO_4)_2$.* 750 ml. of the above fluid are mixed with 2 g. of $Ca_3(PO_4)_2$ gel, acidified to *p*H 6·8 and after 10 min. standing filtered through Chardin folded filter. The clear and faintly coloured fluid obtained has 2·13 mg. dry weight per ml.; 2 μg./E.U.; $A = 500$; 800,000 E.U.; 0·12 % Zn.

(5) *Fractional adsorption on alumina Cγ.* The above fluid was cooled and mixed with 35 ml. of alumina Cγ gel containing 30 mg. dry substance per ml. The alumina gel is centrifuged off, giving a yellow-green cake *A*. The supernatant fluid is mixed with another 35 ml. of alumina gel, adjusted to *p*H 7 and the gel centrifuged off, giving a bluish green cake *B*. To the supernatant colourless fluid is added cooled alcohol, bringing its concentration to 30 % and 30 ml. of alumina gel. The mixture is adjusted to *p*H 6·7 and the gel centrifuged off, giving the colourless cake *C*. The supernatant fluid which is completely inactive is discarded.

(6) *Elution of alumina Cγ gels.* Each of the above three alumina gel cakes is eluted twice with $M/10$ alkaline phosphate, the first time with 40 ml. and the second time with 20 ml. The combined elutions of each cake are dialysed separately against distilled water, giving three phosphate-free clear solutions with the following properties:

A. 60 ml.; 6·6 mg. dry weight per ml.; 5 μg./E.U. $A = 200$; 79,200 E.U.

B. 60 ml.; 4·7 mg. dry weight per ml.; 1·2 μg./E.U. $A = 833$; 235,000 E.U.; 0·15–0·16 % Zn.

C. 60 ml.; 3·8 mg. dry weight per ml.; 0·6 μg./E.U. $A = 1666$; 380,000 E.U.; 0·264 % Zn.

Of these fractions, *A* and *B* contain large concentrations of copper (0·04–0·1 % Cu or more) and are therefore suitable for the isolation of haemocuprein, giving 50–80 mg. of this compound in the crystalline state. Fraction *C*, which contains only traces of Cu but is rich in Zn, is most suitable for further purification of carbonic anhydrase.

(7) *Further purification on alumina Cγ.* Fraction *C* was treated once more with alumina Cγ added in sufficient amount to adsorb the whole enzyme. The gel is centrifuged off, eluted with alkaline phosphate and dialysed. This step gave 150 mg. of the final product with the following properties: 0·45 μg./E.U.; $A = 2222$; 333,000 E.U.; 0·31–0·33 % Zn. The nitrogen content of this preparation was 14·95 %.

B. *Method II*

(1) *Extraction with alcohol and chloroform.* The red blood corpuscles of 2 l. of defibrinated ox blood are washed as in the previous method. 1150 ml. of corpuscles collected are plasmolysed with 575 ml. distilled water, cooled and treated with 575 ml. alcohol and 575 ml. chloroform, both previously cooled in ice. The mixture is carefully stirred, left standing for 15 min. and centrifuged. The cake of denatured protein is discarded and the fluid filtered through a Chardin filter.

(2) *Dialysis of alcohol-chloroform extract.* The collected clear fluid is dialysed for 24 hr. against running water giving 2250 ml. of clear yellowish solution which has 1·72 mg. dry weight per ml.; 2·6 μg./E.U.; $A = 384$; 1,488,000 E.U.

(3) *Total precipitation with ammonium sulphate.* The above fluid is saturated with ammonium sulphate (750 g./l.), the precipitate is collected on a Büchner filter, suspended in a small amount of water, dialysed against running water until free from ammonium sulphate and centrifuged. This gives 110 ml. of brown opalescent fluid containing 17 mg. dry weight per ml.; 1·7 μg./E.U.; $A = 588$; 1,100,000 E.U.

(4) *Fractional precipitation with ammonium sulphate.* The fluid is mixed with 31 g. ammonium sulphate giving 45% saturation. The precipitate (*a*) is filtered off, the filtrate is saturated with ammonium sulphate and filtered again. The second precipitate (*b*) is collected while the filtrate is discarded.

Both precipitates are dissolved in water and dialysed, giving two solutions:

(*a*) 55 ml. of brown and opalescent solution containing 275,000 E.U.

(*b*) 85 ml. of clear and slightly coloured solution containing 9 mg. dry weight per ml.; 1–1·2 μg./E.U.; $A = 830$–1000; 765,000 E.U.; 0·15–0·17% Zn.

(5) *Purification with alumina Cγ gel.* The fraction (*b*), which is still slightly coloured, is treated three times with successive portions of 5 ml. (= 100 mg.) of alumina Cγ at *p*H 6·8 and centrifuged, the cakes being discarded each time. The solution becomes completely colourless although it contains most of the enzyme.

(6) *Fractional precipitation with ammonium sulphate.* The colourless solution is treated with ammonium sulphate to 50% saturation and the precipitate is filtered off and discarded. The filtrate is saturated with ammonium sulphate, the precipitate collected, dissolved in water and dialysed against distilled water until completely free from salt. 50 ml. of the fluid collected have 4·5 mg. dry weight per ml.; 0·45 μg./E.U.; $A = 2220$; 500,000 E.U.; 0·33% Zn. 2 l. of ox blood by this method give therefore 225 mg. of this highly purified enzyme preparation. The yield of this fraction from 2 l. of ox blood in other preparations varied from 96 to 415 mg.

VI. Other preparations of carbonic anhydrase from erythrocytes

Other preparations of carbonic anhydrase from ox blood gave the fractions with the following activities and Zn contents:

(1) $A = 1000$; 0·162% Zn;
(2) $A = 1375$; 0·2% Zn;
(3) $A = 1825$; 0·295% Zn.

As regards other animals the best results were obtained with the red blood corpuscles of sheep which give, if anything, a better yield than the erythrocytes of the ox and a final preparation which had 0·42 μg./E.U.; $A = 2380$; 0·34% Zn.

Attempts at the purification of carbonic anhydrase from the red blood corpuscles of man gave so far a preparation containing only 0·164% Zn which corresponds to about 50% of our purest enzyme.

VII. Purification of carbonic anhydrase from gastric mucosa

It was recently shown by Davenport [1939] that the parietal cells of the gastric mucosa of cats and rats are very rich in carbonic anhydrase which probably plays an important role in the mechanism of formation of HCl. This discovery made possible an attempt at the purification of carbonic anhydrase from a new and unexpected source, thus checking some of our results obtained from the study of the enzyme isolated from erythrocytes. After a rapid survey of the gastric mucosa of different animals we found that by far the best material for

isolation of this enzyme is presented by the mucosa of the pig's stomach. The greatest difficulty in handling such a material is due to the presence of a very large amount of mucus which prevents in this case the application of methods used for isolation of carbonic anhydrase from erythrocytes. We had, therefore, to devise for this material an entirely different method which will be described presently.

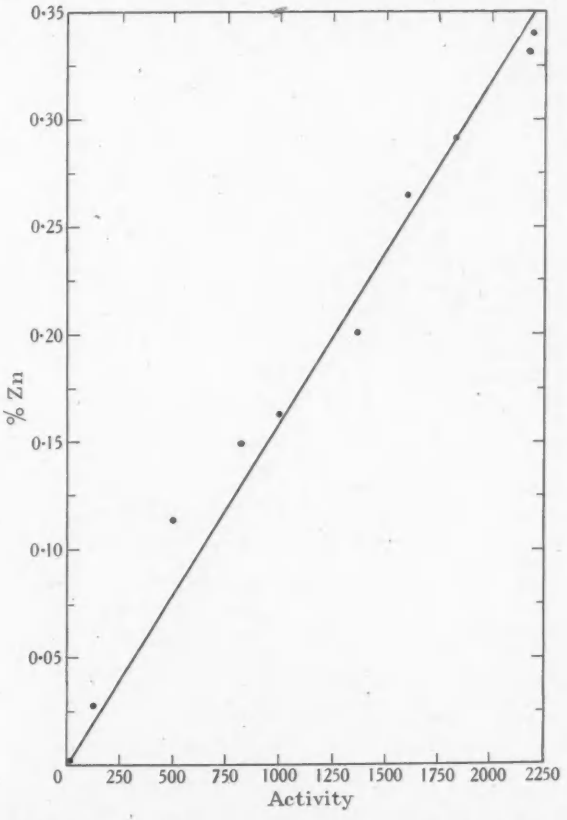

Fig. 1. Relationship between activities (as defined by Meldrum & Roughton) and Zn contents of different fractions of carbonic anhydrase obtained from blood.

(1) *Extraction of enzyme from gastric mucosa.* The stomachs of five pigs are split open and the mucosa is separated from the rest of the tissue by means of sharp scalpels. The mucous membranes thus obtained are minced, giving 1 kg. of wet tissue which is mixed with sand and 150 g. $Na_2SO_4, 10H_2O$ and ground in a mechanical mortar for 15 min. The thick mass is mixed with 2 l. of 15% sodium sulphate and the grinding continued for another 30 min. The fluid collected is centrifuged, sand and coarse particles of tissue are discarded, leaving 2 l. of turbid

solution containing 500,000 E.U. of carbonic anhydrase. From the haemoglobin content of this solution we could easily calculate that the amount of enzyme in this solution which derives from the erythrocytes represents less than 3750 E.U. —in other words, about 0·75 % of the total enzyme content.

(2) *Treatment with barium acetate.* The above solution is treated with 30 % barium acetate until all sulphate is precipitated; the precipitate is centrifuged off, leaving 2 l. of a pink, clear fluid containing 400,000 E.U.

(3) *Fractional precipitation with acetone.* 2 l. of cold solution are mixed with 1 l. of cold acetone and 10 ml. 50 % $CaCl_2$. The precipitate is centrifuged off, while the supernatant fluid is treated with 2 l. of cold acetone. The precipitate is collected and dissolved in 500 ml. of water, giving a distinctly pink solution which contains 11 mg. dry weight per ml.; 18 μg./E.U.; $A = 55$; 305,000 E.U.

This solution is completely free from haemoglobin and its strong pink colour is due entirely to cytochrome *c*.

(4) *Adsorption on tricalcium phosphate.* The solution is mixed with 2 g. tricalcium phosphate gel adjusted to *p*H 6·8 and centrifuged. The cake is eluted with *M*/10 alkaline phosphate and dialysed, giving 360 ml. of clear solution containing 2·2 mg. dry weight per ml.; 7·1 μg./E.U.; $A = 141$; 111,500 E.U.

(5) *Fractional adsorption on alumina Cγ.* The above solution is mixed with 20 ml. alumina Cγ, adjusted to *p*H 7·2, the gel is centrifuged off and discarded, while the supernatant pinkish yellow fluid is mixed with 60 ml. alumina gel adjusted to *p*H 6·8 and centrifuged. The gel is collected, eluted with *M*/10 alkaline phosphate and dialysed, giving 160 ml. of solution containing 2·6 mg. dry weight per ml.; 4 μg./E.U.; $A = 250$; 104,000 E.U.

(6) *Precipitation with acetone.* The above solution is cooled in ice and treated with two volumes of cold acetone. The precipitate is redissolved in 17 ml. water, giving a clear solution containing 7·5 mg. dry weight per ml.; 1·5 μg./E.U.; $A = 667$; 85,000 E.U.; 0·12–0·13 % Zn.

Further purification of the enzyme from this preparation was not successful although this stage was reached several times. This difficulty may be partly due to some impurities which cannot easily be removed, partly to the fragility of the enzyme caused by the initial stages of purification.

If we consider our best preparation of carbonic anhydrase from erythrocytes as a pure enzyme the preparation obtained from gastric mucosa will be only 30 % pure. It shows, nevertheless, the corresponding high concentration of Zn, and this gives additional evidence supporting the view that carbonic anhydrase is a Zn-protein compound.

VIII. Tests for other metals in preparations of carbonic anhydrase

In addition to Zn the concentration of several other metals was estimated in various fractions of carbonic anhydrase and this gave the following results:

Magnesium was estimated by the titan yellow method. 80 mg. of enzyme showing 0·57 μg./E.U. and $A = 1750$ were found to contain only 0·006 % Mg which indicates that it is present only as an impurity.

Manganese, estimated colorimetrically by the permanganate method in a sample of 30 mg. of the same enzyme preparation, was found to be present only in traces.

Iron, estimated by $\alpha\alpha'$-dipyridyl in preparations of different activities, gave the results summarized in Table 1.

Table 1

Preparations	Activity	% Fe
I	250	0·007
II	1000	0·005
III	2250	0·003

These results show clearly that the very small amounts of Fe found in different preparations can only be considered as impurities.

Copper. Very interesting results were obtained with the estimation of copper in different fractions of carbonic anhydrase preparations. As is shown in Fig. 2,

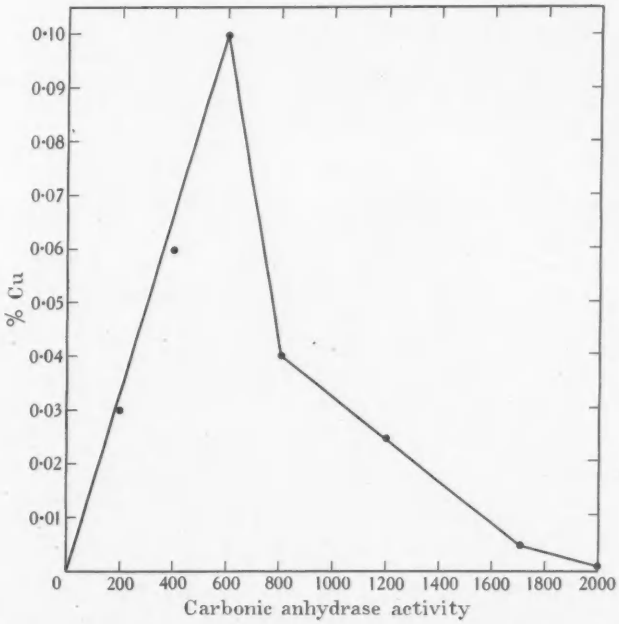

Fig. 2. Relationship between activities and Cu contents of different fractions of carbonic anhydrase.

the concentration of Cu rises gradually with the increase in the activity of the enzyme, reaching almost 0·1% and then suddenly drops almost to vanishing point. The initial increase in Cu concentration is due to haemocuprein which accompanies carbonic anhydrase during the first few steps of purification. A point is, however, reached when haemocuprein is almost quantitatively separated from carbonic anhydrase and this point is indicated by the sudden drop in Cu concentration in the fraction containing carbonic anhydrase. This observation may serve as a warning against any rash conclusion as to the nature of an enzyme based on a parallelism between the activities and concentrations of one of the substances found in some fractions during the first steps of purification.

Lead. 35 mg. of pure enzyme tested with the dithizone method was found to be completely free of lead.

The above tests show that purified carbonic anhydrase which contains about 0·33 % Zn is either devoid of other metals such as Fe, Cu, Mn, Pb and Mg or contains them as impurities in very low concentrations.

IX. Some properties of carbonic anhydrase

A. *Effect of inhibitors*

It was already shown by Meldrum & Roughton [1933] that carbonic anhydrase is strongly inhibited by KCN, H_2S and NaN_3[1]. We have confirmed this observation and found that a concentration of cyanide even as low as $4 \times 10^{-6} M$ inhibits carbonic anhydrase to the extent of almost 85 %. The inhibitory effect of cyanide in spite of being so very marked is completely reversible. For instance, 37 mg. of half-purified enzyme preparation ($A = 1100$) in 10 ml. water are mixed with 1 ml. M KCN at pH 6·5 and after standing 30 min. at room temperature the mixture is saturated with ammonium sulphate, the precipitate centrifuged off, dissolved in water and dialysed. The preparation after this treatment recovers at least 90 % of its original activity.

B. *Stability of the enzyme*

Enzyme preparations when purified keep well in solution. It is, however, important to note that when the enzyme is inactivated by long standing or by various treatments Zn remains bound to the protein and cannot be removed by dialysis. While the separation of Zn from enzyme protein by acids irreversibly destroys its activity, the inactivation of the enzyme does not necessarily liberate the metal. This shows that the surplus of Zn found in certain fractions of enzyme preparations is due to the presence in these fractions of a certain amount of inactivated enzyme which still retains its complete share of Zn. The activities of these fractions when plotted against their Zn contents always give points lying above the line of strict proportionality (see Fig. 1).

X. Evidence of Zn-protein nature of carbonic anhydrase

The evidence upon which our main conclusion is based, namely that carbonic anhydrase is a Zn-protein compound, can be summarized as follows:

(1) The facts that the enzyme is very strongly inhibited by KCN, H_2S and NaN_3, that this inhibition is instantaneous and perfectly reversible, clearly indicate that the active group of the enzyme must be a metal.

(2) The highly purified enzyme preparation contains 0·31–0·33 % Zn. Such concentration of metal in a protein is already of the same magnitude as that of Fe in haemoglobin or of Cu in polyphenol oxidase, haemocyanin or in crystalline haemocuprein.

(3) There is a distinct proportionality between the activities of enzyme preparations and their Zn contents (Fig. 1).

(4) Purification of carbonic anhydrase by two entirely different methods gives final products of the same high activity and the same Zn content.

(5) Enzyme preparations obtained from two very different sources such as the red blood corpuscles and the gastric mucosa have Zn contents corresponding to their catalytic activities.

[1] Since this paper went to press we have been able to demonstrate (Mann & Keilin [1940], *Nature, Lond.*, **146**, 164) that carbonic anhydrase is strongly and reversibly inhibited by sulphanilamide and some other sulphonamide compounds. These inhibitors, unlike KCN, H_2S and NaN_3, are highly specific and therefore will render it possible to determine experimentally the function of carbonic anhydrase in respiration and in the secretion of HCl by gastric mucosa.

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(6) Other metals which are known to occur in biological material, such as Fe, Cu, Mn, Pb, Mg and others, are either absent from the highly purified enzyme preparation, or found only in negligible concentrations.

Zn in carbonic anhydrase forms therefore an active part of its prosthetic group stoichiometrically combined with the protein. That this Zn does not derive from outside as a result of a selective accumulation of this metal during purification of the enzyme is shown by the fact that several other proteins (serum- and egg-albumin, haemoglobin, haemocuprein and laccase) submitted to somewhat similar purifications have been found to be either free from Zn or to contain less of it than their crude preparations.

It is important to note that carbonic anhydrase is the only Zn compound which has characteristic catalytic properties. Other substances such as inorganic and simple organic Zn compounds, Zn-porphyrin, crystalline Zn-insulin and Zn-protein compounds of serum and different tissues are completely devoid of this catalytic property. This is not surprising considering that other metals such as Fe and Cu form with different proteins compounds either acting as carriers of molecular oxygen or capable of catalysing a series of completely different reactions.

XI. Degree of purity of the final product

The best fraction of carbonic anhydrase obtained by Meldrum & Roughton [1933] was already a highly active preparation corresponding probably to about 65–75 % pure enzyme.

The claim by Kiese & Hastings [1940] to have obtained an enzyme preparation which is several times more active than the above preparation cannot, therefore, be accepted. In fact, the high activity values obtained by these workers are misleading and probably due to the low temperature (5 instead of 15°) at which their estimations were carried out. Considering that the temperature coefficient of the non-catalysed reaction is much higher than that of the catalysed reaction [Meldrum & Roughton, 1933] the relative activities of enzyme preparations can be compared only when determined at the same temperature. In fact, while at 15° about 0·45 μg. of our purest enzyme preparation doubled the velocity of the non-catalysed reaction, at 5° this effect is already obtained by 0·12 μg. of the same preparation.

If, however, the activity values are obtained at different temperatures, they should be supplemented by values for the catalytic activity of the plasmolysed red blood corpuscles, obtained under exactly the same conditions. The ratio between these values determines independently of the temperature the stage of purification reached by the enzyme preparation.

Such data show for instance that the activity of our purest enzyme preparation is only about 150 times higher than that of the red blood corpuscles, which suggests that the concentration of the enzyme in the red blood corpuscles is very high.

It is difficult to determine with certainty the degree of purity of our most active enzyme preparation. Several considerations, however, indicate that our final product is either pure or almost pure enzyme. Thus, the purification of the enzyme from different sources and by different methods gives the same final product of which neither the activity nor Zn content could be increased by any further attempt at purification. The high Zn content of the final product leaves very little room for the presence of another protein not combined with Zn. Finally, the cataphoretic experiments in the Tiselius apparatus carried out at the Low Temperature Research Station (see Appendix, p. 1176) show that our

purified preparation behaves like a homogeneous protein. The examination of different fractions collected after these experiments reveals, moreover, a distinct proportionality between their activities and their nitrogen and Zn contents.

The experiments in an ultracentrifuge carried out by Dr F. Eirich of the Colloid Science Laboratory, Cambridge, show that the enzyme is of approximately half the size of a haemoglobin molecule.[1] This would indicate that the protein in each molecule of enzyme is combined with two atoms of Zn.

XII. The role of Zn in organisms

The presence of Zn in tissues of plants and animals was discovered by Lechartier & Bellamy [1877]. Since this discovery a large amount of work has been done on the distribution, localization and estimation of Zn in tissues of different organisms under normal, experimental and pathological conditions. It is now well established that Zn is a true and general microconstituent of living organisms. Its concentration in higher animals has a constant value for each organism and for each of its tissues[2] and this value is not much affected even by a marked change in the amount of Zn taken with food. Although the presence of Zn in living organisms has been known for more than 60 years there was no indication as to its biological significance, nor even a suspicion as to its possible connexion with a definite physiological function of an organism.

In fact Eichholtz [1934], after reviewing the literature on Zn in connexion with living organisms, concluded that "we do not know of any substance in organisms of warm-blooded animals the biological significance of which is due to the presence of zinc, and we do not know of any physiological function which requires the co-operation of zinc."

The discovery of Zn in carbonic anhydrase thus establishes for the first time the physiological function of this metal in organisms.

Iron, copper and zinc are three widely distributed metals, the biological significance of which is now definitely established.

The fact that the distribution of Zn in nature is much wider than that of carbonic anhydrase shows only that Zn, like iron and copper, forms with proteins several compounds having different properties and functions.

XIII. Concentration of three metallo-protein compounds in erythrocytes

The comparison of the catalytic activities and Zn contents in the red blood corpuscle and in the highly purified enzyme shows that probably the whole of the Zn present in the red blood corpuscles belongs to carbonic anhydrase (Table 2).

Table 2

	Pure enzyme	Red blood corpuscle	
	a	*b*	*a*/*b*
Activity	2220	14·3	154
% Zn	0·33	0·00245	137

It is therefore possible to determine the concentration of carbonic anhydrase in the red blood corpuscles and to compare it with those of the other two metallic constituents of erythrocytes. The results thus obtained, summarized in Table 3,

[1] The detailed account of these experiments will be published separately.

[2] The data concerning the distribution of Zn in the red blood corpuscles and plasma in man and higher animals are given in a paper by Burstein [1929].

Table 3

Constituents of the red blood corpuscles	Amount in g. (dry wt.) per 100 ml. corpuscles	Metal in the compound %
Haemoglobin	28·00	0·34 Fe
Carbonic anhydrase	0·21	0·33 Zn
Haemocuprein	0·06	0·34 Cu

show that the concentration of carbonic anhydrase in the red blood corpuscles is three to four times higher than that of haemocuprein and about 133 times lower than that of haemoglobin. One litre of mammalian blood contains approximately 1 g. of carbonic anhydrase.

Summary

1. Carbonic anhydrase is an enzyme discovered by Meldrum & Roughton [1933] in the red blood corpuscles of mammals, which catalyses both phases of the reaction $H_2CO_4 \rightleftharpoons CO_2 + H_2O$. In mammals high concentration of this enzyme is also found in the gastric mucosa [Davenport, 1939].

2. Methods are described for purification and isolation of this enzyme from the red blood corpuscles and the gastric mucosa of mammals.

3. The purest enzyme preparation obtained from erythrocytes of ox and sheep by two distinct methods is a colourless protein containing 14·95% N and about 0·33% Zn. It is devoid of haematin, Fe, Cu, Mn, Mg and Pb. Evidence is brought forward that this preparation is either pure or almost pure enzyme. Its catalytic activity is about 150 times higher than that of the red blood corpuscles.

4. The maximum yield of this purified product is about 200 mg./l. of ox blood.

5. That carbonic anhydrase is a Zn protein compound where Zn forms the active part of the enzyme molecule is strongly supported by the following considerations:

(*a*) An immediate, strong and completely reversible inhibition of this enzyme by small concentrations of KCN, H_2S and NaN_3 which are known to react in this way only with metals.

(*b*) The presence of 0·33% Zn in the purest preparations obtained from erythrocytes of ox or sheep by two very different methods.

(*c*) The presence of Zn in carbonic anhydrase obtained from gastric mucosa.

(*d*) The absence of other metals from purified enzyme preparation.

(*e*) The proportionality between the enzyme activity and Zn content of different fractions obtained from erythrocytes and gastric mucosa.

6. The concentration of carbonic anhydrase in red blood corpuscles is very high. 100 ml. of erythrocytes contain about 0·21 g. of the purified product, which is three to four times higher than the concentration of haemocuprein and about 133 times lower than that of haemoglobin.

7. The presence of Zn in carbonic anhydrase establishes for the first time the physiological function of this metal in organisms.

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APPENDIX

CATAPHORETIC BEHAVIOUR OF CARBONIC ANHYDRASE

By E. C. Bate Smith

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research, Cambridge

A preparation of purified carbonic anhydrase was submitted by Prof. Keilin and Dr Mann for examination in the Tiselius apparatus. The solution, containing 0·5% of protein (0·33% of Zn, 0·45μg./E.U., $A = 2222$) was dialysed for 18 hr. against 0·05M phosphate buffer, pH 7·0. In the cataphoresis cell the preparation showed a *single* boundary moving at a velocity of $-1{\cdot}9 \times 10^{-5}$ cm./sec./volt/cm. Dialysed against buffer at pH 8·0 the velocity was $-2{\cdot}2 \times 10^{-5}$.

A second preparation (0·55μg./E.U., $A = 1820$) was examined at pH 5·8. This was less pure than the first preparation and, in fact, showed two boundaries. The main boundary, corresponding to the carbonic anhydrase component, moved at this pH with a velocity of $-0{\cdot}85 \times 10^{-5}$ cm./sec./volt/cm. Extrapolating these values to zero velocity, the enzyme would appear to be isoelectric in the neighbourhood of pH 5, confirming the observation of Kiese & Hastings [1939].

139. ALCOHOL ENZYME OF *BACT. COLI*

By JACK LESLIE STILL

From The Biochemical Laboratory, Cambridge

(*Received 1 July 1940*)

Cell-free extracts of *Bact. coli* were found to contain a highly active alcohol enzyme requiring coenzyme I. This investigation deals with the properties of this enzyme. These are very similar to those of the alcohol enzyme isolated by Negelein & Wulff [1937] from yeast and would suggest the probable identity of the two enzymes.

I. *Preparation of the enzyme*

The organism used was *Bact. coli commune* Escherich (National Collection of Type Cultures No. 86). It was grown and maintained on agar to which was added tryptic digest of casein and inorganic salts. The cells from 30 hr. growth on 30 Roux bottles were washed off the medium with distilled water and centrifuged. The thick suspension of cells was ground for 2 hr. in the wet-crushing mill [Booth & Green, 1938]. The suspension of crushed cells, volume about 40 ml., was diluted with 60 ml. water and centrifuged for 40 min. at 3000 r.p.m. The supernatant was heated to 55°; cooled immediately and centrifuged for 45 min. at 12,000 r.p.m. The clear yellow supernatant fluid was then dialysed overnight against running water. Such preparations had a dry weight of 8–10 mg. per ml., and when kept on ice retained activity for several weeks.

The activity of the enzyme was measured anaerobically by the Thunberg tube technique, and manometrically with Barcroft and Warburg manometers.

II. *Components of the system*

The enzyme did not reduce methylene blue in the presence of ethyl alcohol. On addition of coenzyme I reduction took place (Table 1).

Table 1. *Demonstration of alcohol dehydrogenase in extract of* Bact. coli

	Additions to tubes (ml.)			
	1	2	3	4
Enzyme extract	0·5	0·5	0·5	0·5
Phosphate buffer *M*/15 *p*H 7·4	1·0	1·0	1·0	1·0
Methylene blue 0·5%	0·1	0·1	0·1	0·1
Ethyl alcohol 9·7%	0·1	0·1	0·1	—
Coenzyme I (14% pure) 5 mg./ml.	0·2	—	0·2	0·2
Semicarbazide 2 *M*	0·2	0·2	—	0·2
Water	0·5	0·7	0·7	0·6
Reduction time in min.	$1\frac{5}{6}$	120	$2\frac{1}{4}$	65

Treatment of the enzyme solution in such a way as to destroy coenzyme dehydrogenase diminished the rate of reduction of methylene blue by the enzyme. Addition of milk flavoprotein partially restored this loss in activity (Table 2). The enzyme was precipitated with 5 vol. of acetone and filtered. The precipitate, after washing with acetone and ether, was ground in water and the suspension dialysed overnight against running water. It was then heated at

Table 2. *Effect of coenzyme dehydrogenase on acetone preparation*

	Q_{MB}
Enzyme solution	63·3
Acetone powder	5·3
Acetone powder + milk flavoprotein	25·3

Q_{MB} = μl. hydrogen transferred to methylene blue per hr. per mg. dry weight of enzyme preparation.

52° for 10 min. and finally centrifuged. The supernatant was then tested under the standard conditions. As a source of coenzyme dehydrogenase milk flavoprotein was used [Corran & Green, 1938].

III. *Reaction with molecular oxygen*

The system alcohol enzyme, alcohol, coenzyme I and coenzyme dehydrogenase can react with molecular oxygen in the presence of a suitable carrier such as methylene blue. In this reaction a ketone fixative is also required (Table 3). In experiments designed to show the complete oxidation of a known amount of ethyl alcohol to acetaldehyde, the O_2 uptake was never theoretical. There was always a small O_2 uptake in the absence of the substrate. If this blank reaction is assumed to be suppressed in the presence of ethyl alcohol, the observed O_2 uptake would agree with theory (Fig. 1).

Table 3. *Aerobic oxidation of ethyl alcohol by the enzyme*

	Additions (ml.) in Barcroft manometers				
	1	2	3	4	5
Enzyme	1·0	1·0	1·0	1·0	1·0
Phosphate $M/15$ *p*H 7·4	1·0	1·0	1·0	1·0	1·0
Methylene blue 0·5%	0·5	0·5	0·5	—	0·5
Coenzyme I (14% pure) 5 mg./ml.	0·2	—	0·2	0·2	0·2
Ethyl alcohol 10%	0·3	0·3	0·3	0·3	—
Semicarbazide 2 M	0·2	0·2	—	0·2	0·2
Water	—	0·2	0·2	0·5	0·3
O_2 uptake in μl./10 min.	168	0	54	11	0

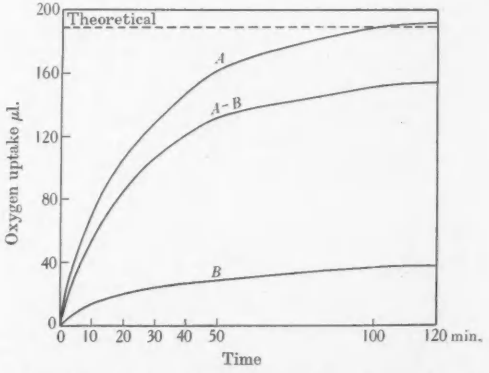

Fig. 1. Oxidation of ethyl alcohol to acetaldehyde by enzyme. Manometer *A* contained 1·0 ml. enzyme, 1·0 ml. $M/15$ phosphate buffer *p*H 7·4, 140 μg. coenzyme I, 0·2 ml. 2 M semicarbazide, 0·4 ml. 0·5% methylene blue and 0·1 ml. 1% ethyl alcohol. Total volume 3·2 ml. Manometer *B* contained no alcohol. Temperature 38°.

IV. *Kinetics*

Fixatives. Semicarbazide, cyanide and hydrazine accelerated the reduction of methylene blue anaerobically. Hydroxylamine was inhibitory. However, cyanide and hydrazine accelerated the blank reduction in the absence of substrate. Accordingly semicarbazide was used where a fixative was required.

*p*H. The optimum *p*H for the reduction of methylene blue by alcohol in the presence of the enzyme was found to be 7·4 (Table 4). Borate appeared to inhibit the activity of the enzyme.

Table 4. *Effect of* p*H on activity*

In Thunberg tubes containing enzyme 0·25 ml., coenzyme I (14% pure) 0·5 mg., buffer *M*/15 2·0 ml. and 0·5% methylene blue 0·1 ml.

*p*H	Reduction time in min.	*p*H	Reduction time in min.
5·8	75	7·0	9¾
6·4	37	7·4	3
6·8	12¼	7·75	4¼

Concentrations of substrate, coenzyme I and carrier. The relation of the rate of oxidation to the concentrations of substrate, coenzyme I and methylene blue is shown in Table 5 and Figs. 2 and 3.

Table 5. *The effect of concentration of ethyl alcohol*

In Barcroft manometers. 1·0 ml. enzyme, 1·0 ml. phosphate buffer *p*H 7·4, 0·2 ml. semicarbazide 2 *M*, 0·5 ml. methylene blue 0·5%, 1·0 mg. coenzyme I (14% pure coenzyme) and ethyl alcohol. Total volume 3·2 ml. Temperature 38°.

Alcohol concentration (*M*)	O_2 uptake in μl./25 min.
0	9
0·007	47
0·022	63
0·074	149
0·22	141

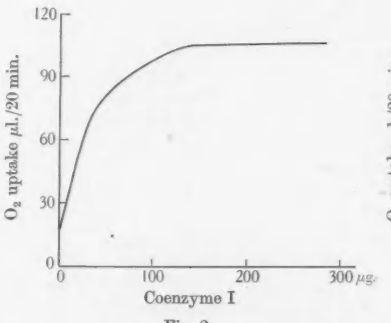

Fig. 2.

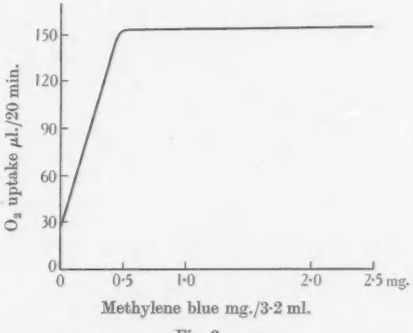

Fig. 3.

Fig. 2. Effect of concentration of coenzyme I. Barcroft manometers containing 1·0 ml. enzyme, 1·0 ml. *M*/20 phosphate buffer *p*H 7·4, 0·2 ml. 2 *M* semicarbazide, 0·5 ml. 0·5% methylene blue, and 0·3 ml. 10% ethyl alcohol (in both cups). Total volume 3·2 ml. Temperature 38°.

Fig. 3. Effect of concentration of carrier. Barcroft manometers containing 1·0 ml. enzyme, 1·0 ml. *M*/20 phosphate buffer *p*H 7·4, 0·2 ml. 2 *M* semicarbazide, coenzyme I 141 µg., 0·3 ml. 10% ethyl alcohol (in both cups). Total volume 3·2 ml. Temperature 38°.

V. *Specificity of substrate*

The enzyme oxidizes readily the lower aliphatic alcohols. Methyl alcohol is oxidized only slowly (Table 6).

Table 6. *Various alcohols as substrate*

Using Thunberg tubes containing 1·0 ml. enzyme, 1·0 ml. phosphate buffer $M/15$ *p*H 7·4, 0·1 ml. methylene blue 0·5%, 0·2 ml. semicarbazide 2 M, 1·0 mg. coenzyme I (14% pure coenzyme) and substrate 0·1 ml.

Substrate	Reduction time in min.
—	60
Methyl alcohol	17
Ethyl alcohol	$1\frac{5}{6}$
Propyl alcohol	$1\frac{5}{6}$
n Butyl alcohol	2
Amyl alcohol	5
Methyl and ethyl alcohols	2
Glycerol	52

VI. *Mechanism of reaction*

Coenzyme I and coenzyme dehydrogenase have been shown to be components of the system. By analogy with the yeast enzyme one would expect the coenzyme I to undergo a cycle of reduction by alcohol and oxidation by acetaldehyde. This was found to be the case with the bacterial enzyme also.

Reduction of coenzyme I. 1·5 ml. enzyme, 0·2 ml. semicarbazide, 1·0 ml. $M/20$ phosphate buffer *p*H 7·4, 0·1 ml. 97% ethyl alcohol, 2·2 ml. water and 5·0 mg. coenzyme I (14% pure coenzyme) were incubated in a Thunberg tube for 30 min. at 37°. Similar tubes, one without cozymase and one with enzyme previously boiled, were also incubated. The contents of the tubes were brought to boiling and filtered. 3·0 ml. of the filtrate in each instance were made up to 4·5 ml. with water and examined in the Hilger "Spekker" apparatus [cf. Dewan & Green, 1937]. For the complete system $\log I_0/I$ at 345 mμ was 1·7. The control with boiled enzyme gave $\log I_0/I = 0{\cdot}5$ at 345 mμ.

Reoxidation of reduced coenzyme I. Gale & Stephenson [1939] showed that malic dehydrogenase is extracted from *Bact. coli* by crushing and resides mainly in the liquid fraction of the crushed cells. It survives further treatment and occurs in the enzyme solution used in the present investigation. Reduced coenzyme I was prepared by means of this enzyme. 1·5 ml. of enzyme, 2·0 ml. $M/20$ phosphate buffer *p*H 7·4, 1·0 ml. water, 0·4 ml. 2 M potassium cyanide, 1·1 ml. M sodium malate and 15 mg. coenzyme I (14% pure coenzyme) were incubated in a Thunberg tube for 30 min. A similar tube without coenzyme I was also set up. The contents of both tubes were boiled and filtered. The filtrate was diluted 1 in 4 and examined as above. $\log I_0/I = 1{\cdot}7$ at 345 mμ.

The reoxidation of reduced coenzyme I by the alcohol enzyme was demonstrated as follows: 1·5 ml. enzyme, 1·25 ml. water, 1·25 ml. reduced coenzyme I and 1·0 ml. M acetaldehyde were added to a Thunberg tube. Two other tubes, one without reduced coenzyme I and one with boiled enzyme, were also set up. The tubes were incubated for 30 min. at 38°, the contents boiled and filtered. The filtrate was examined as before. For the complete system $\log I_0/I = 0{\cdot}2$ at 345 mμ. The control with boiled enzyme gave $\log I_0/I = 1{\cdot}4$ at 345 mμ.

VII. *The product of oxidation*

The product of oxidation was isolated from a reaction mixture made up of the following: 23 ml. enzyme, 10 ml. 2*M* semicarbazide, 25 ml. *M*/15 phosphate buffer, 50 mg. coenzyme I (14% pure) and 1·3 ml. 96% ethyl alcohol. This mixture was incubated at 38° for 2 hr. during which it was aerated with a stream of oxygen. Protein was removed with colloidal iron and methylene blue removed by filtration through kieselguhr. 1 g. 2:4-dinitrophenylhydrazine was added to the filtrate together with enough HCl to make the final concentration normal. The solution became opalescent immediately. After 24 hr. standing at room temperature a yellow precipitate was filtered off and recrystallized once from absolute alcohol and twice from ethyl acetate and ligroin. The yellow crystals obtained gave M.P. 161° decomp. The known derivative from acetaldehyde melted at 160° with decomposition. Mixed melting point 160°. *Analysis* (Weiler): C=44·27%; H=3·42%; N=25·40%. Calculated for $C_8H_8N_4O_4$: C=44·44%; H=3·70%; N=25·93%.

VIII. *Inhibition by iodoacetate*

Dixon [1937] found that *M*/1000 iodoacetate inhibits completely the yeast alcohol dehydrogenase. Lutwak-Mann [1938] found that the aerobic oxidation of ethyl alcohol by *Acetobacter suboxydans* was unaffected by prolonged incubation with *M*/100 iodoacetate and the anaerobic oxidation only slightly affected. In the case of the cell-free enzyme of *Bact. coli* incubation with *M*/500 iodoacetate for 5 min. caused 75% inhibition of the activity.

IX. *Oxidation of alcohol by washed suspensions of* Bact. coli

Cook & Stephenson [1928] observed that a strain of *Bact. coli* oxidized acetate, the O_2 consumption being $\frac{3}{4}$ of that required for the complete oxidation to CO_2. The organism did not oxidize ethyl alcohol or acetaldehyde under the same conditions. Dismutation of acetaldehyde was observed.

Table 7 shows that washed suspensions of the organism used in this investigation oxidized alcohol anaerobically. Aerobically, the washed suspensions took up O_2 in the presence of phosphate and either ethyl alcohol or acetaldehyde. Dismutation of acetaldehyde in bicarbonate-CO_2 buffer at *p*H 7·4 could not be observed.

Table 7. *Demonstration of alcohol oxidation by washed suspensions of* Bact. coli

	Additions to tubes (ml.)			
	1	2	3	4
Bacterial suspension 9 mg./ml. dry weight	1·0	1·0	1·0	1·0
Phosphate buffer *M*/15 *p*H 7·4	1·0	1·0	1·0	1·0
Methylene blue 0·5%	0·2	0·2	0·2	0·2
Ethyl alcohol 97%	0·1	0·1	0·1	—
Coenzyme I (14% pure) 5 mg./ml.	0·2	0·2	—	0·2
Semicarbazide 2*M*	0·2	—	0·2	0·2
Water	—	0·2	0·2	0·1
Reduction time in min.	$1\frac{3}{4}$	$2\frac{1}{4}$	2	150

The addition of coenzyme I to suspensions of whole cells made no appreciable difference to the rate of reduction of methylene blue in the presence of alcohol. *Bact. coli* has been shown to contain coenzyme I [Gale & Stephenson, 1939].

SUMMARY

1. A cell-free preparation of alcohol dehydrogenase has been made from *Bact. coli commune*.

2. The enzyme oxidizes ethyl alcohol in the following system: ethyl alcohol—alcohol dehydrogenase—coenzyme I—coenzyme dehydrogenase—methylene blue—oxygen.

3. The effect of variation in the concentration of these components, the specificity of substrate and the effect of fixatives have been investigated.

4. The reoxidation of reduced coenzyme I by the enzyme in the presence of acetaldehyde demonstrates that the enzyme is reversible.

5. The product of the oxidation is acetaldehyde which has been isolated as the 2:4-dinitrophenylhydrazone.

6. The enzyme is inhibited by iodoacetate.

7. The whole organism oxidizes both ethyl alcohol and acetaldehyde. The cell-free preparation does not oxidize acetaldehyde.

The author is grateful to Dr D. E. Green and Dr V. H. Booth for advice and to Dr M. Stephenson for her interest in this work. The wet-crushing mill was provided by a grant from the Medical Research Council to this laboratory.

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140. THE ISOLATION OF SMALL QUANTITIES OF GLUCOSAMINE AND CHONDROSAMINE

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(*Received 29 June 1940*)

The widespread occurrence of amino-sugars as components of polysaccharides is becoming increasingly evident. The conclusion that an amino-sugar is present in a material under investigation has, however, in many instances, been based exclusively on the appearance of a positive colour reaction when the products of acid hydrolysis of the material under investigation are warmed with an alkaline solution of acetylacetone and subsequently treated with Ehrlich's *p*-dimethylaminobenzaldehyde reagent according to the method of Elson & Morgan [1933] or one of the modifications of this method [Nilsson, 1936; Palmer *et al.* 1937; Sørensen, 1938; Hewitt, 1938]. For purposes of preliminary investigation a positive colour may be accepted as a very strong indication that an amino-sugar is present, but this evidence should be confirmed by the isolation of the hydrochloride or some other suitable crystalline derivative of the amino-sugar before its presence in the material is finally accepted. The methods available for the isolation of small amounts of the naturally occurring amino-sugars, especially when these substances are contaminated with sugars or with the products of protein hydrolysis, cannot be considered satisfactory. A useful procedure for the isolation of glucosamine is that developed by Chargaff & Bovarnick [1937] in which the amino-sugar is converted into the sparingly soluble carbobenzyloxyglucosamine which yields the free hexosamine on hydrogenation according to Bergmann & Zervas [1932]. This method does not appear to be suitable for the isolation of small quantities (10–20 mg.) of glucosamine, especially in presence of excess of other sugars or amino-acids. Furthermore, the carbobenzyloxy-derivative of chondrosamine has been found by us to be considerably more soluble in water than carbobenzyloxyglucosamine and is therefore quite unsuitable for the isolation of the former amino-sugar.

Irvine & Earl [1922] showed that glucosamine formed a sparingly soluble Schiff's base when treated in alkaline solution with salicylaldehyde and somewhat later Bergmann & Zervas [1931] described the corresponding anisaldehyde derivative. During an attempt to isolate the amino-sugar that was believed to be present in the specific polysaccharide of *Bact. dysenteriae* (Shiga) a number of similar Schiff's bases were prepared and examined [Morgan, 1938]. Of these only the *p*-nitrobenzylidene and the cinnamylidene derivatives were found to be suitable for the isolation of small quantities of glucosamine. The corresponding derivatives of chondrosamine, owing to their solubility in water, were of little use for the isolation of this amino-sugar. Neuberger [1938] employed the Schiff's base derived from 2:4-dihydroxybenzaldehyde for the isolation of glucosamine from the polysaccharide component of serum albumin. Unfortunately 2:4-dihydroxybenzylidenechondrosamine is also too soluble in water to be used for the isolation of small amounts of chondrosamine.

The ease with which Schiff's bases of this kind can be formed and subsequently decomposed with liberation of the amino-sugar in almost theoretical yield prompted us to investigate the properties of Schiff's bases derived from other aldehydes in the hope of finding a derivative that would enable small amounts (10–30 mg.) of glucosamine and chondrosamine to be isolated in good yield from crude protein or polysaccharide hydrolysis products.

The following aldehydes were employed: (1) *p*-nitrobenzaldehyde, (2) *p*-nitrocinnamic aldehyde, (3) 3-methoxy-4-hydroxybenzaldehyde (vanillin), (4) 2-hydroxynaphthaldehyde, (5) 2:4-dinitrobenzaldehyde, (6) 2-hydroxy-4-benzeneazobenzaldehyde (benzeneazosalicylaldehyde), (7) 2-hydroxy-4-*p*-nitrobenzeneazobenzaldehyde, (8) 3-methoxy-4-acetoxybenzaldehyde, (9) 2-methoxynaphthaldehyde, (10) 2-hydroxy-*p*-nitrobenzeneazonaphthaldehyde.

The first four aldehydes reacted readily with glucosamine and gave Schiff's bases that were sparingly soluble in water. Aldehydes (3) and (4) gave rise to sparingly soluble derivatives of chondrosamine as well as of glucosamine; (8) and (9) failed to react with the amino-sugars under the conditions employed whereas (10) was so insoluble in the usual organic solvents that its solution could only be accomplished when inconveniently large volumes of solvent were employed. The Schiff's base, 2-hydroxy-4-benzeneazobenzylidenechondrosamine derived from aldehyde (6), was too soluble in water to be of use.

Experimental

The method employed for the preparation of the various Schiff's bases was similar in all the examples given and for this reason the experimental details will only be described in one instance.

2-Hydroxynaphthylideneglucosamine. A solution of glucosamine hydrochloride (20 mg.) in 0·5 ml. water is treated with sodium acetate (2 mol.) and gently shaken until the solid has dissolved. The mixture at this stage should be just acid to litmus. An excess of 2-hydroxynaphthaldehyde (50 mg.), prepared according to Duff & Bills [1934], is dissolved in methyl alcohol (3 ml.) and is added to the amino-sugar. After thorough mixing and keeping for 2–3 hr. in a dark place, the solution, which develops a bright yellow colour, is taken to dryness *in vacuo* at 10–15°. The excess aldehyde is then removed from the reaction products by extraction with dry chloroform or ether in which the Schiff's base of the amino-sugar is insoluble. After removing the remaining chloroform the residue is extracted at 0° with three 0·5 ml. portions of distilled water in order to remove inorganic salts. It is essential that this process should be carried out as near to 0° as possible as at higher temperatures the Schiff's base becomes appreciably soluble in water. The three washings are mixed, concentrated to about 0·5 ml. and set aside at 0° for several hours. A further amount of the Schiff's base usually separates in the form of bright yellow crystals and is collected in the bottom of a centrifuge tube in the usual manner. This additional quantity of base, after thoroughly washing with ice-cold water, is added to the main portion. The material, which consists of almost pure 2-hydroxynaphthylideneglucosamine, is dried *in vacuo* and recrystallized from 1 to 2 ml. of warm methyl alcohol; the Schiff's base separates in tufts of fine needles, M.P. 202–203° (decomp.), and the yield, which at this stage is about 70%, can be increased to 90–95% by the cautious addition of acetone to the alcoholic solution. The substance mutarotates; the initial $[\alpha]_{5461}$ +274° (in methyl alcohol) changing to +217° in 18 hr. (Found: C, 61·10; H, 5·71; N (Dumas), 4·15%. $C_{17}H_{19}O_6N$ requires C, 61·26; H, 5·71; N, 4·20%.) The substance is soluble in methyl alcohol and in pyridine, slightly soluble in cold ethyl alcohol and acetone

and insoluble in ether or chloroform. In aqueous alcoholic suspension the Schiff's base, when treated in the cold with a few drops of conc. HCl, dissolves and a few minutes later separates in the form of a crystalline hydrochloride. The yellow needles sinter at about 200° but do not melt. (Found: Cl, 9·27%. $C_{17}H_{22}O_7NCl$ requires Cl, 9·15%.)

2-Hydroxynaphthylidenechondrosamine. M.P. 175–178° (decomp.). Somewhat more soluble in methyl and ethyl alcohols than the corresponding glucosamine derivative. Initial $[\alpha]_{5461}+287°$ (in methyl alcohol) changing to $+258°$ in 18 hr. (Found: C, 59·80; H, 5·63; N (Dumas), 4·20%. $C_{17}H_{19}O_6N$ requires C, 61·26; H, 5·71; N, 4·20%.) The hydrochloride of the Schiff's base did not separate from solution as described above for the glucosamine compound.

p-*Nitrobenzylideneglucosamine.* Colourless crystals from ethyl alcohol, M.P. 182–184° (decomp.). (Found: C, 50·5; H, 5·33; N (Dumas), 9·05, 9·16%. $C_{13}H_{16}O_7N_2$ requires C, 50·00; H, 5·13; N, 8·97%.) Sparingly soluble in water. Insoluble in acetone, ether and chloroform.

p-*Nitrobenzylidenechondrosamine.* Colourless crystals from ethyl alcohol, M.P. 175–176° (decomp. 177°). (Found: C, 49·83; H, 5·24; N (Dumas), 9·02%. $C_{13}H_{16}O_7N_2$ requires C, 50·00; H, 5·13; N, 8·97%.)

3-Methoxy-4-hydroxybenzylideneglucosamine. Colourless crystals, M.P. 184° (decomp.). Sparingly soluble in all usual solvents. $[\alpha]_{5461}+64°$ (in pyridine). For analysis the substance was dissolved in pyridine and precipitated with ether. (Found: C, 53·78; H, 6·04; N (Dumas), 4·51%. $C_{14}H_{19}O_7N$ requires C, 53·67; H, 6·07; N, 4·48%.)

3-Methoxy-4-hydroxybenzylidenechondrosamine. M.P. 153–155°. (Found: C, 52·96; H, 6·4; N (Dumas), 4·73%. $C_{14}H_{19}O_7N$ requires C, 53·67; H, 6·07; N, 4·48%.)

p-*Nitrocinnamylideneglucosamine.* M.P. 187° (decomp.). Crystallized from pyridine-ether. Initial $[\alpha]_{5461}+57·6°$ (in pyridine) changing to $+41·5°$ overnight. (Found: C, 53·61; H, 5·33; N (Dumas), 8·29%. $C_{15}H_{18}O_7N$ requires C, 53·26; H, 5·30; N, 8·28%.)

p-*Nitrocinnamylidenechondrosamine.* M.P. 172–173°. (Found: C, 53·41; H, 5·58; N (Dumas), 8·15%. $C_{15}H_{18}O_7N$ requires C, 53·26; H, 5·30; N, 8·28%.)

All melting points are uncorrected. The C and H determinations were made by Drs Weiler and Strauss of Oxford.

In a series of comparable experiments the yields of the different Schiff's bases were determined. The results of some of these experiments are shown in Table 1. It will be seen that with the exception of *p*-nitrocinnamylidenechondrosamine all the Schiff's bases examined were suitable when amounts of amino-sugar as large as 50 mg. were employed. Experience showed, however, that when less than this was used the yield in most instances diminished rapidly with decreasing amount of amino-sugar. Vanillin and 2-hydroxynaphthaldehyde, however, were found to give excellent yields of the Schiff's base even when the amount of amino-sugar used fell to 10 mg. The vanillin derivatives were colourless whereas the corresponding 2-hydroxynaphthaldehyde bases were bright yellow in colour. The sparing solubilities of 2-hydroxynaphthylideneglucosamine and -chondrosamine suggested the use of these compounds as the basis of a method for the isolation of glucosamine and chondrosamine from mixtures containing simple sugars and amino-acids.

The influence of foreign substances upon the final yield of amino-sugar

Some experiments were carried out to determine the influence of certain sugars on the yield of the amino-sugars when these were separated by means of

Table 1. *The yields of Schiff's bases*

Amino-sugar	Amount of amino-sugar hydrochloride mg.	Aldehyde employed	Yield of Schiff's base %
Glucosamine	100	*p*-Nitrocinnamaldehyde	85, 81
	50	*p*-Nitrobenzaldehyde	85
	50	3-Methoxy-4-hydroxybenzaldehyde	85, 90, 92
	50	2-Hydroxy-1-naphthaldehyde	96, 90
	10	,, ,,	76
Chondrosamine	50	*p*-Nitrocinnamaldehyde	39
	50	*p*-Nitrobenzaldehyde	63, 70
	40	3-Methyl-4-hydroxybenzaldehyde	53, 58
	45	2-Hydroxy-1-naphthaldehyde	84, 79
	30	,, ,,	85, 88
	20	,, ,,	78

Table 2. *Showing the influence of simple carbohydrates on the yield of amino-sugars*

Amino-sugar hydrochloride employed	Wt. mg.	Carbohydrate added	Amino-sugar hydrochloride recovered Yield (%)
Glucosamine	30	Galactose (20 mg.) + fructose (22 mg.)	70
	10	Galactose (5 mg.) + fructose (5 mg.)	73
	10	Galactose (10 mg.)	72
	5	Galactose (5 mg)	42
Chondrosamine	30	Galactose (33 mg.)	57*
	30	Galactose (7 mg.) + fructose (8 mg.)	84
	20	Galactose (24 mg.)	65*
	20	Galactose (5 mg.) + fructose (5 mg.)	74
	10	Galactose (5 mg.) + fructose (5 mg.)	72
	10	Galactose (10 mg.)	72

* Schiff's base and amino-sugar hydrochloride collected by filtration.

the 2-hydroxynaphthylidene derivative. The results of a few of these experiments are given in Table 2. It will be seen that even a large excess of added sugar has no appreciable influence on the yield of amino-sugar finally obtained. Other experiments showed that 50 mg. of glucosamine and chondrosamine could be recovered in 80 and 73% yield respectively after admixture with 200 mg. of a mixture made up from 50 mg. of each of the following sugars: glucose, fructose, rhamnose and galactose.

The influence of an amino-acid mixture on the yield of the amino-sugar hydrochloride has also been investigated. Under the experimental conditions described the amino-acids form Schiff's bases with 2-hydroxynaphthaldehyde but the separation of these compounds from the Schiff's base of the amino-sugar is easily accomplished owing to the ready solubility of the amino-acid derivatives in chloroform. The Schiff's bases of the amino-sugars are insoluble in this reagent. The influence of the amino-acids on the separation is shown in Table 3. In these experiments an amino-acid mixture, derived from the acid hydrolysis of gelatin, was added in amount equivalent to 50% of the quantity of amino-sugar employed in the determination. It will be seen that this quantity of amino-acid mixture has no appreciable influence on the final yield of amino-sugar hydrochloride. Considerably larger quantities of amino-acids caused the yield to decrease but this falling off in yield could be partly checked by using as solvent for the amino-acid Schiff's bases a mixture of chloroform and carbon

Table 3. *Showing the influence of protein hydrolysis products on the yield of amino-sugars*

Amino-sugar hydrochloride employed	Wt. mg.	Hydrolysed gelatin added mg.	Amino-sugar hydrochloride recovered Yield (%)
Glucosamine	40	20	91
	30	15	77
	20	10	82
	10	5	70
	30	50*	61
Chondrosamine	40	20	73
	30	15	75
	20	10	70

* Hydrolysed casein

tetrachloride (3 : 1) in place of pure chloroform. One experiment using a casein hydrolysis product is also given. The purity of the amino-sugar hydrochloride finally isolated was checked in most of the experiments by the determination of N and optical rotation.

Application

A few experiments were undertaken involving the isolation and identification of the amino-sugar from biological products which contained both sugars and amino-acids.

The following method was found to be satisfactory. The material under investigation is hydrolysed with HCl or H_2SO_4 until the amount of amino-sugar, as estimated by the colorimetric method, reaches a maximum. About 20–30 mg. of amino-sugar are usually sufficient for the method as here described. The acid is almost completely removed by repeated evaporation *in vacuo* at room temperature or by means of baryta according to the nature of the acid employed. If considerable darkening has occurred during the hydrolysis the slightly acid products are treated with 5–10 mg. of charcoal and filtered through a thin layer (1–2 mm.) of charcoal on a small conical filter. The filter is then washed three times with a few drops of distilled water and the filtrate and washings are concentrated to a syrup *in vacuo* in a pointed centrifuge tube measuring about $12 \times 1{\cdot}5$ cm. The walls of the tube are finally washed down with about 0·5 ml. of distilled water. From this stage in the process to the final isolation of the amino-sugar hydrochloride the various steps in the method are all carried out in the centrifuge tube. The acidity of the syrup is now reduced, by means of added $KHCO_3$ if necessary, until the solution no longer reacts blue with Congo red. The solution must at no time react blue with litmus paper. Sodium acetate crystals (150–200 mg.) are now added and dissolved in the solution by gentle shaking. The 2-hydroxynaphthaldehyde is added in considerable excess in the form of a solution in methyl alcohol (3–4 ml.). The excess of aldehyde employed must depend on the amount of amino-acids present in the mixture but four or five times the quantity calculated for the amino-sugar present can safely be added. The contents of the centrifuge tube are now thoroughly mixed and set aside in a cool, dark place for 2–3 hr. The solution, which becomes bright yellow during this period, is evaporated at room temperature *in vacuo* to about 0·5 ml. The semi-solid mass is treated with a few drops of methyl alcohol and set aside at 0° overnight. The contents of the centrifuge tube are now taken to dryness *in vacuo* below 20° and the solid mass thoroughly extracted three times with about 6 ml. of freshly distilled chloroform. This process removes the excess aldehyde and the Schiff's bases of the amino-acids and leaves behind the Schiff's

base of the amino-sugar and the inorganic salts. The addition of 2 ml. of freshly distilled ether to the chloroform extract before centrifuging will enable the insoluble Schiff's base of the amino-sugar to be packed down readily in the point of the centrifuge tube. Without the addition of ether the insoluble material tends to float to the top of the tube. The extracted residue is now freed from chloroform and treated at 0° with 1 ml. of cold distilled water. The inorganic salts dissolve and the Schiff's base of the amino-sugar remains as a bright yellow residue. The water is left in contact with the Schiff's base for several hours at 0° and in all three or four extractions are made. The combined aqueous extracts are concentrated to 1·0–1·5 ml. and again put aside at 0° for several hours. A further quantity of the Schiff's base which separates is removed by centrifuging, washed three times at 0° with distilled water and added to the main preparation. The crude Schiff's base, which usually melts about 10° lower than the pure compound, can now be recrystallized from warm methyl alcohol in order to identify the amino-sugar or it can be directly converted into the amino-sugar hydrochloride. This can readily be accomplished by suspending the Schiff's base in 1 ml. of 4*N* HCl and heating the centrifuge tube in a boiling water bath for 15 min. The yellow colour of the Schiff's base is destroyed and the white 2-hydroxynaphthaldehyde separates from solution. The free aldehyde is removed from the cooled solution by extraction with ether, three 5 ml. portions of ether being sufficient for this purpose. The combined ether extracts are then shaken with 1 ml. distilled water and the aqueous extract is added to the main acid solution of the amino-sugar hydrochloride. The acid solution is then freed from excess ether, centrifuged to remove any insoluble particles and the clear aqueous solution is transferred to a clean centrifuge tube by means of a capillary pipette. The residue is washed with two 1 ml. portions of water. The solution and washings are concentrated to a syrup, one drop of 2*N* HCl and several drops of methyl alcohol are added and the mixed solution is treated with acetone until a slight cloudiness appears. The solution is then set aside for several hours at 0°. Under these conditions glucosamine and chondrosamine hydrochlorides separate from solution in an almost pure condition and in excellent yield. A single recrystallization from aqueous ethyl alcohol usually gives a pure product.

By means of the above method glucosamine has been isolated in good yield and identified as the amino-sugar present in the ecto-skeleton of the tail segments of the Egyptian scorpion *Buthus quinquestriatus*.

From 150 mg. of polysaccharide (kindly supplied by Dr Neuberger), isolated from crystalline egg albumin, it has been possible to isolate 76% of the amino-sugar that is present according to colorimetric estimation. The amino-sugar was identified as glucosamine hydrochloride. Neuberger [1938] has already described the isolation of about 60% of the amino-sugar estimated to be present in the polysaccharide by the colorimetric method and has identified the substance as glucosamine hydrochloride.

The method has enabled the chondrosamine hydrochloride derived from 100 mg. of a preparation of the calcium salt of chondroitin sulphuric acid to be isolated in pure crystalline condition and in a yield of 54% of the amount determined by the colorimetric method. The Schiff's base gave the following analytical figures: M.P. 172–174° $[\alpha]_{5461}+258°$ (after 24 hr.). N, 4·3%. $C_{17}H_{19}O_6N$ requires N, 4·20%. The amino-sugar hydrochloride gave $[\alpha]_{5461}+108°$ changing to +99·6° after 18 hr. According to Jorpes and Bergström [1936] 50 g. of chondroitin sulphuric acid (2·08% N) gave 61% yield of chondrosamine hydrochloride when the method of direct crystallization of the hydrochloride was employed.

Discussion

The application of the method that has been described to the isolation of glucosamine and chondrosamine will probably throw some light on the failure to isolate more than a small part of the amino-sugar estimated to be present in many biological products by the colorimetric method. Observations of this nature have been reported by Neuberger [1938] who could isolate 60% only of the glucosamine estimated to be present in the polysaccharide of crystalline egg-albumin. Morgan [1938] failed to isolate any amino-sugar from the specific polysaccharide of *Bact. dysenteriae* (Shiga) although the colorimetric method indicated that at least 20% of the total polysaccharide was composed of amino-sugar. By means of the Schiff's base described in the present paper a part of the amino-sugar in the specific polysaccharide has now been isolated and identified as glucosamine; further details of this work will be given in another paper. More recently, Landsteiner & Harte [1940] obtained a yield of glucosamine that represented only 47% of the amino-sugar contained in the polysaccharide component of the mucin of hog's gastric mucosa. When taken together, these and other similar observations might be considered to suggest that a substance other than glucosamine and chondrosamine exists which gives rise to a positive colour reaction when treated with Ehrlich's reagent under the conditions laid down for the quantitative test [Elson & Morgan, 1933], and indeed, the work of Miles & Pirie [1939] gives some support to this idea. It may be, however, that in some instances at least, the poor yields of amino-sugar isolated are due solely to the difficulty of liberating and isolating small quantities of glucosamine and chondrosamine by the methods employed. The nature of the component sugars and the rates of hydrolysis of the different polysaccharide complexes are in most instances largely unknown. For this reason it is clearly essential that the exact conditions which give complete hydrolysis must be determined for each polysaccharide molecule investigated and that after hydrolysis the amino-sugar must be completely free from combination with its neighbouring sugar molecules and any substituents in the amino-group, such as acetyl or formyl, must be completely eliminated. If either of these hydrolytic processes is incomplete all methods that depend on the sparing solubility of a Schiff's base for the isolation of an amino-sugar will give poor yields and such results cannot be used as evidence for the existence of an unknown amino-sugar. When complete hydrolysis has been achieved there seems no reason why 70–80% yields of the glucosamine or chondrosamine should not be obtained in spite of the presence of sugars and amino-acids.

Summary

A method is described for the isolation of small quantities (10–30 mg.) of glucosamine and chondrosamine. The method depends on the sparing solubility in water of the Schiff's bases, 2-hydroxynaphthylidene-glucosamine or -chondrosamine. The presence of sugars and amino-acids does not appreciably decrease the yield of Schiff's base.

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141. THE ELIMINATION OF 3:4-BENZPYRENE FROM THE ANIMAL BODY AFTER SUBCUTANEOUS INJECTION

I. UNCHANGED BENZPYRENE

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(*Received 8 July 1940*)

Evidence has already been presented [Chalmers, 1940] that 3:4-benzpyrene after intravenous injection of its collodial solution is eliminated from the rat as a derivative having a blue fluorescence in the ultra-violet beam. Spectrographic examination showed that this fluorescence was characterized by two diffuse bands in the blue region of the spectrum. It appears that this fluorescent derivative of benzpyrene is eliminated in the bile and subsequently in the faeces, and to a much smaller extent in the urine. In order to obtain a sufficient quantity of the substance for complete identification, recourse was had to subcutaneous injections of benzpyrene, using a 1% solution in olive oil; by this means much larger quantities of the hydrocarbon could be made available for metabolism than was possible by the intravenous route. That the same derivative was obtained in the excreta as with the former method of injection was shown by the similarity of the fluorescence spectrum in organic solvents, the solubility in NaOH solution and the selective adsorption on alumina.

Qualitative elimination

The urine and faeces collected separately from the rats, which were kept in metabolism cages, were extracted by the method already described [Chalmers, 1940]. In this method, after removal of fatty material, the fluorescent substance was separated from other benzene-soluble constituents of the urine or faeces by chromatographic adsorption on alumina. When a benzene solution obtained from fluorescent urine was passed through a tower of alumina, it was observed that the filtrate, though colourless in daylight, had a violet fluorescence in the ultra-violet beam. The fluorescent derivative of benzpyrene is strongly adsorbed on alumina from benzene solution, and it therefore appeared that the filtrate contained a second fluorescent fraction. Spectrographic examination of this filtrate revealed the presence of the characteristic fluorescence spectrum bands of benzpyrene.

Although it seemed unlikely that the result could be explained by the accidental contamination of the urine with benzpyrene from the hair of recently injected animals, it was thought advisable to examine the fluorescent elimination products obtained from a source not liable to such contamination. An operation for the daily collection of bile has been devised by Dr P. R. Peacock, and will be described elsewhere in detail. It consists essentially of the introduction into the gall-bladder of a glass cannula connected by narrow rubber tubing to a rubber balloon of about 25 ml. capacity, situated in the subcutaneous tissues. From this balloon, bile can be aspirated at intervals, through the skin,

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by means of a syringe and small hypodermic needle. Fowl bile collected in this way after daily intravenous injections of 5 ml. of 0·03% benzpyrene colloid was extracted, after acidification with HCl, with *n*-butyl alcohol. The solution was evaporated to dryness in N_2 under reduced pressure and taken up in benzene. The procedure was then the same as for the urine extracts. Spectrographic analysis revealed the presence of benzpyrene, thus establishing qualitatively the elimination in fowl bile of unchanged benzpyrene after intravenous injection. Subsequently benzpyrene was detected by the same method in an extract of fowl excreta, after the subcutaneous injection of the hydrocarbon. The elimination of unchanged benzpyrene in the excreta of two fowls injected intravenously with benzpyrene colloid has been reported by Peacock [1936]. This carcinogen is therefore, to some extent, eliminated unchanged by the fowl after intravenous or subcutaneous injection.

The original method of extracting the fluorescent rat faeces was unsuitable for the isolation of unchanged benzpyrene, since it involved passage through 2*N* NaOH to remove fat, and consequent disappearance of any benzpyrene present. It was found possible to remove most of the non-fluorescent material by extracting with warm acetone the residue obtained after evaporating the original alcoholic extract to dryness. The acetone solution was filtered after thorough chilling in the refrigerator to about 4°. Two further extractions yielded practically all the fluorescent material, and the combined extracts were transferred to benzene solution and subjected to chromatographic analysis. Two fluorescent zones could be seen on the chromatogram: an upper, having a greenish-yellow fluorescence, and a lower, having a violet fluorescence. The initial yellow filtrate had a faint green fluorescence, and was discarded. The chromatogram was developed with ether, which rapidly eluted the violet-fluorescent zone. The presence of benzpyrene in this eluate was detected by fluorescence spectrum analysis.

Quantitative elimination

In order to find what fraction of the injected benzpyrene was eliminated unchanged from the rat, three adult rats were injected subcutaneously, each with 2 ml. of a 1% solution of benzpyrene in olive oil. The animals were housed in a metabolism cage, and the urine and faeces collected separately in the usual way. Ethanol extracts of samples of the faeces were examined each day for the presence of fluorescent elimination products. The intensity of the fluorescence of the samples decreased progressively until, after about one month, the samples were practically non-fluorescent. There were by this time no palpable signs of residual injections to be found. It was concluded that most of the benzpyrene had been eliminated, although examination in the ultra-violet beam of animals injected subcutaneously one month previously with a solution of benzpyrene in olive oil, showed that the tissue at the site of injection was intensely fluorescent, indicating that some of the benzpyrene had been encapsulated there.

The pooled samples of the urine and faeces, respectively, were extracted by the modified method already indicated. In the case of the faecal extract, on filtration of the benzene solution through an alumina tower a wide zone, having a greenish-yellow fluorescence, was formed at the top of the tower, and another wide zone, having a violet fluorescence, was formed lower down. The initial yellow filtrate which was non-fluorescent was discarded. The filtrate from development with ether, containing the eluate of the violet-fluorescent zone, was collected. This filtrate had a pale yellow colour in daylight, and an intense violet fluorescence in the ultra-violet beam.

In the case of the urine extract, on filtration of the benzene solution through an alumina tower a wide zone containing a yellow-fluorescent material was formed at the top, but there was no obvious violet-fluorescent zone below. However, on development of the chromatogram with ether, a colourless filtrate with a violet fluorescence was obtained.

Spectrographic analysis. The apparatus used for recording of fluorescence spectra has been described [Chalmers & Peacock, 1936]. Ilford Iso-zenith photographic plates were used. Quantitative estimation of the amount of benzpyrene present was made by comparison of the intensity of fluorescence of the solutions of unknown strength with that of standard solutions photographed on the same plate, as described by Berenblum & Kendal [1936]. It was found that there was a recognizable variation in the intensity of fluorescence of 0·1, 0·2, and 0·3 mg./100 ml. solutions of benzpyrene in ethanol under the conditions used, and comparisons of ethanol solutions of the recovered benzpyrene with these standards have been made in the experiments described here. It was found that with solutions of benzpyrene containing more than 0·3 mg./100 ml. the intensity of fluorescence did not appear to increase so markedly with increase in concentration, and such solutions were therefore less suitable as standards.

The violet-fluorescent filtrate from the chromatogram of the faecal extract described above was taken to dryness. A solution of the residue in ethanol was then prepared for spectrographic analysis. Solvent was added until the solution had a fluorescence of intensity comparable on visual examination with that of the standards. On spectrographic examination, it was found that the faecal extract had a fluorescence corresponding to a total benzpyrene content of 0·4–0·6 mg. Since, in all, 60 mg. of benzpyrene were injected, the recovery represented about 1% of the amount administered.

The fluorescence spectrum of the urine extract revealed the presence of only a trace of benzpyrene.

Efficiency of the method of extraction. In order to establish the efficiency of the method of extracting the benzpyrene from the faeces of the rat, a sample was taken from the pooled ethanol extracts of the faeces collected daily from a series of rats injected subcutaneously with benzpyrene in olive oil. The sample was divided into two fractions, A and B. To A were added 2 mg. benzpyrene; fraction B served as a control. Both fractions were then extracted by the acetone method already described. On spectrographic analysis, it was found that the extract from fraction A contained approximately 2 mg. benzpyrene, while the control extract from B contained about 0·2 mg. Hence it follows that 90% of the added benzpyrene was recovered by this method of extraction, and that the method could therefore be relied on to yield reasonably quantitative data.

Discussion

The primary aim of a series of experiments at present in progress in this laboratory, in which rats have been injected subcutaneously with a solution of benzpyrene in olive oil at weekly intervals, has been the isolation of the fluorescent derivative of benzpyrene eliminated in the faeces and urine under such conditions. The fluorescent derivative of benzpyrene eliminated in the faeces in the course of the quantitative experiment described in this communication has been collected, and it is hoped that the amount eliminated may be estimated by fluorescence analysis. It appeared, from previous work, that a relatively large amount of the injected benzpyrene is converted into this derivative, but

about 1% of the benzpyrene injected in this quantitative experiment has been recovered unchanged in the faeces. The detection of unchanged benzpyrene in fowl excreta indicates that the elimination of a small fraction of the injected hydrocarbon unchanged after intravenous or subcutaneous injection is not peculiar to the rat.

After the subcutaneous injection of benzpyrene in the rat, it is probable that unchanged benzpyrene is slowly taken up by the blood stream and transferred to the liver, where most of it is converted into the fluorescent derivative. A small quantity of the hydrocarbon, however, is apparently eliminated from the liver unchanged or in conjugation with bile salts. The quantity of benzpyrene present at any time in the bile of the rat must be small, since, in the quantitative experiment, less than 0·2 mg. benzpyrene was eliminated per rat in one month. The fact that benzpyrene dissolves in aqueous sodium deoxycholate to the extent of 2 mg. per ml. [Winterstein & Vetter, 1934], suggests that the unchanged benzpyrene may be eliminated in the bile as a conjugate with bile salts. The presence of bile salts does not influence the fluorescence spectrum of benzpyrene [Chalmers, 1938]. This finding is in agreement with the results of Lorenz [1935], who photographed the absorption spectra of the hydrocarbon choleic acids made by Fieser & Newman [1935] and found that these spectra and those of the unchanged hydrocarbons were identical, showing that the choleic acids were completely dissociated.

In the case of the fowl, evidence has been found of the presence of a fluorescent derivative of benzpyrene and of unchanged benzpyrene in the bile after intravenous injection. In the case of the rat, it has not been practicable to examine the fluorescent bile, but the presence of the fluorescent derivative of benzpyrene and of unchanged benzpyrene in the faeces indicates that the mechanism of elimination of benzpyrene is the same. Only a trace of benzpyrene was found in the urine in the quantitative experiment, and while in the larger scale experiment the presence of benzpyrene became more evident, the elimination of benzpyrene in the urine appears to be of minor importance.

The mode of administration of the benzpyrene may have some influence on the relative amounts of the fluorescent derivative of benzpyrene and of unchanged benzpyrene eliminated. After intravenous injection of benzpyrene colloid, or subcutaneous injection of benzpyrene in olive oil, the quantity of the fluorescent derivative eliminated seems to be greater than the quantity of unchanged benzpyrene. But it has been noted that bile obtained from a fowl injected intravenously with a 0·05% solution of benzpyrene in "Emmal" oil emulsion contained a relatively larger amount of unchanged benzpyrene, while the amount of fluorescent derivative present was less than after the injection of a colloidal solution of the hydrocarbon owing, possibly, to the whole of the injected benzpyrene reaching the liver in a shorter space of time. After subsequent injection of benzpyrene colloid in the same bird, the bile contained a trace of unchanged benzpyrene and a relatively large amount of the derivative, similar to the bile obtained after the initial injection of the colloid.

Summary

Evidence has been obtained that after the subcutaneous injection of benzpyrene in the rat, a small fraction of the hydrocarbon is eliminated unchanged in the urine and faeces. Approximately, 1% of 60 mg. benzpyrene injected subcutaneously in three rats was eliminated unchanged in the faeces, while only a trace was eliminated in the urine.

Evidence has also been obtained of the partial elimination of benzpyrene in an unchanged form in fowl bile after intravenous injection, and in fowl excreta after subcutaneous injection.

The mechanism of elimination of benzpyrene by the liver has been discussed.

One of the authors (A. H. M. K.) is indebted to the British Empire Cancer Campaign for a personal grant.

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142. EXPERIMENTS ON THE CHEMOTHERAPY OF CANCER

4. FURTHER EXPERIMENTS WITH ALDEHYDES AND THEIR DERIVATIVES

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(*Received 18 July 1940*)

STRONG [1938; 1939] has shown that the growth of spontaneous mammary tumours in mice is inhibited by feeding heptaldehyde. This was confirmed by Boyland & Mawson [1938] who found, however, that citral had a greater inhibitory effect than heptaldehyde. Some other aldehydes have now been examined but none has proved to be as effective as citral. In an attempt to understand the action of these aldehydes on tumours a number of related compounds and possible metabolic products have been tested; of these malonic acid was found to have marked inhibitory action.

An attempt has been made to put the experiments on a quantitative basis so that the activities of compounds can be more accurately compared and the significance of the results assessed.

EXPERIMENTAL

Grafted tumours

The toxicity of each substance was determined and it was given daily by forced feeding to groups of five Dilute Brown mice grafted with sarcoma M.C.D.B.I. [cf. Boyland & Boyland, 1939]. Dosing was commenced on the day on which the tumour was grafted. No doses were given to the animals on Sundays. The daily dose chosen was about one-quarter of the L.D. 50 and was suspended or dissolved in 0·5 ml. water with gum. Some compounds had a cumulative toxic action and killed mice when one-quarter of the L.D. 50 was administered repeatedly; in these cases a smaller dose was given.

The tumours were measured with callipers twice weekly and their sizes compared with those of tumours grafted at the same time in similar but untreated mice. The mean rate of growth of the tumours for all the periods between measurements was calculated and compared with the mean growth rate of the tumours in control mice. The percentage inhibition of growth due to the treatment is given in Table 2. The probability (P) that the inhibition is greater than could have arisen by errors of sampling is calculated by the method of t [Fisher, 1936]. If the value of P exceeds 0·05 then the inhibition cannot be considered significant. This does not necessarily imply that the hypothesis that the inhibition is real is not true but that the experiment for which P is calculated cannot be taken by itself as proof of the hypothesis, within the limits of likelihood corresponding to $P=0·05$.

On the whole there is rather poor correspondence between the results with grafted tumours and spontaneous tumours. The effect on the grafted sarcomata is generally smaller but the variations also are smaller and, as the results depend on a larger number of measurements, the effect is often significant.

Sebacic acid, sebacic acid hydrazide, citral, furfuramide and phloroglucinaldehyde were less effective on grafted tumours than on spontaneous tumours; while adipamide, ethyl malonate, malonamide, acrolein, crotonaldehyde, furfuraldehyde and furfuracrylic acid were more effective. Only heptaldehyde (50 mg. per day), malonic acid and citral produced significant inhibition of growth with both types of tumours. These last three compounds must be considered the most effective of the substances described in this paper.

Boyland & Mawson [1938] obtained no inhibition of the Crocker Sarcoma 180 by feeding 50 mg. heptaldehyde per day but a definite inhibition with 5 mg. phloroglucinaldehyde daily.

Primary tumours

As the growth rates of primary tumours vary greatly each tumour must be used as its own control. Induced primary tumours were obtained by injection of 1 mg. methylcholanthrene into stock mice. Induced tumours and spontaneous mammary tumours were measured twice weekly as soon as they were obtained. They were allowed to grow until the sum of two diameters of the tumour had increased by at least 7 mm. and this, the control rate of growth, was expressed in mm. growth per day ((i) in the tables).

The mice were then dosed for 14 days and the rate of growth per day determined ((ii) in the tables). The ratio of these two rates of growth shows the effect of the treatment and from this ratio the inhibition expressed as a percentage of the original growth can readily be obtained. Those cases in which partial retrogression occurred during the treatment are shown by the negative ratio of growth and the inhibition is taken as being over 100%.

The tables show large variations in the response of different mice treated with the same compound. The inhibition obtained in each series was examined according to the methods for treating the difference of means on a unique sample described by Fisher [1936] and the values for t and the probability (P) determined.

EFFECT OF HEPTALDEHYDE ON INDUCED TUMOURS

Heptaldehyde was given in 50 mg. doses daily to mice with primary induced tumours. The treatment had very slight and variable effect on the growth of the tumours, as the results in Table 1 show. Induced tumours like grafted tumours are less susceptible to the action of heptaldehyde than are spontaneous carcinomata.

Table 1. *The ratio of growth rate of induced tumours under treatment with heptaldehyde to their normal growth rate*

In columns A, B, C and D, which refer to separate mice, the numerators of fractions refer to the increase in size of the tumour (as the sum of length and breadth in mm.) while the denominators refer to the number of days required for that growth.

Mouse	A	B	C	D
(i) Growth in control period	$\frac{15}{22}$	$\frac{9}{5}$	$\frac{20}{14}$	$\frac{8}{10}$
(ii) Growth in treatment period	$\frac{12}{14}$	$\frac{6}{14}$	$\frac{18}{14}$	$\frac{18}{14}$
(iii) Ratio $\frac{\text{(ii)}}{\text{(i)}}$	0·69	0·24	0·90	1·60

Average of $\frac{\text{Growth in treatment period}}{\text{Growth in control period}}$ 0·86 or 14% inhibition.

Table 2. *The ratio of growth rate of mouse tumours under treatment to their normal growth rate*

In columns A, B, C and D the numerators in the fractions refer to the increase in size (length and breadth in mm.) of the tumour while the denominators refer to the number of days required for that growth; each column A, B, C and D refers to a separate mouse. If any diminution in size occurred during the treatment period this is recorded by a minus sign and the inhibition is considered as over 100%.

	Toxicity L.D. 50 for mice mg.	Daily dose mg.	Effect on grafted sarcomata M.C.D.B.I. in groups of 5–15 mice		Effect on spontaneous carcinomata (i) Growth in control period. (ii) Growth in treatment period. (iii) Ratio (ii)/(i)												Average effect on spontaneous carcinomata	
					A			B			C			D				
			Inhibition %	Probability (P) that the inhibition is significant	(i)	(ii)	(iii)	(i)	(ii)	(iii)	(i)	(ii)	(iii)	(i)	(ii)	(iii)	Inhibition of growth under treatment %	Probability (P) that the inhibition is significant
Heptaldehyde	500	25	9	0·1	$\frac{8}{22}$	$\frac{4}{14}$	0·78	$\frac{15}{17}$	$\frac{12}{14}$	0·97	$\frac{7}{7}$	$\frac{2}{14}$	0·14	$\frac{12}{7}$	$\frac{17}{14}$	0·71	35	0·2
Heptaldehyde	—	50	20	0·02	$\frac{11}{14}$	$\frac{-2}{14}$	*−0·18*	$\frac{13}{16}$	$\frac{7}{14}$	0·61	$\frac{10}{21}$	$\frac{2}{14}$	0·30	$\frac{14}{7}$	$\frac{8}{14}$	0·29	75	0·02
Heptoic acid	160	25	5	—	$\frac{8}{18}$	$\frac{10}{14}$	1·60	$\frac{10}{14}$	$\frac{12}{16}$	1·05	$\frac{8}{32}$	$\frac{5}{14}$	1·42	$\frac{25}{30}$	$\frac{3}{12}$	0·30	Nil	—
Sebacic acid	150	25	19	0·2	$\frac{10}{13}$	$\frac{4}{14}$	0·37	$\frac{9}{10}$	$\frac{1}{14}$	0·08	$\frac{10}{11}$	$\frac{2}{14}$	0·16	$\frac{9}{11}$	$\frac{4}{14}$	0·35	76	0·01
Sebacic acid dihydrazide	10	2	6	0·2	$\frac{23}{12}$	$\frac{14}{14}$	0·52	$\frac{11}{10}$	$\frac{9}{14}$	0·58	$\frac{13}{12}$	$\frac{5}{11}$	0·42	$\frac{16}{17}$	$\frac{11}{14}$	0·83	41	0·02
Pimelic acid	120	25	6	0·1	$\frac{10}{19}$	$\frac{7}{16}$	0·84	$\frac{7}{12}$	$\frac{8}{16}$	0·86	$\frac{11}{12}$	$\frac{5}{14}$	0·39	$\frac{15}{23}$	$\frac{4}{14}$	0·44	37	0·05
Adipic acid	200	25	0	—	$\frac{9}{9}$	$\frac{4}{14}$	0·29	$\frac{13}{12}$	$\frac{8}{14}$	0·53	$\frac{10}{10}$	$\frac{11}{14}$	0·79	$\frac{12}{18}$	$\frac{9}{14}$	0·97	35	0·1
Adipamide	150	25	28	0·01	$\frac{18}{12}$	$\frac{3}{14}$	0·14	$\frac{7}{11}$	$\frac{11}{14}$	1·22	$\frac{12}{14}$	$\frac{11}{14}$	0·91	$\frac{10}{11}$	$\frac{0}{14}$	0	43	0·3
Mucic acid	200	25	16	0·1	$\frac{9}{20}$	$\frac{4}{14}$	0·63	$\frac{9}{28}$	$\frac{4}{14}$	0·89	$\frac{7}{14}$	$\frac{3}{10}$	0·60	$\frac{9}{8}$	$\frac{10}{14}$	0·63	30	0·05
Glutaric acid	150	25	8	0·5	$\frac{9}{12}$	$\frac{1}{14}$	0·10	$\frac{14}{40}$	$\frac{0}{14}$	0	$\frac{16}{8}$	$\frac{23}{14}$	0·82	$\frac{7}{14}$	$\frac{8}{14}$	1·14	48	0·2
Malonic acid	100	20	32	0·05	$\frac{18}{38}$	$\frac{-6}{14}$	*−0·91*	$\frac{15}{14}$	$\frac{-1}{14}$	*−0·07*	$\frac{18}{34}$	$\frac{2}{14}$	0·27	$\frac{13}{14}$	$\frac{-7}{14}$	*−0·54*	*131*	0·02
Ethyl malonate	160	40	27	0·05	$\frac{6}{14}$	$\frac{4}{14}$	0·66	$\frac{11}{12}$	$\frac{3}{14}$	0·43	$\frac{7}{18}$	$\frac{6}{14}$	1·10	$\frac{8}{13}$	$\frac{7}{14}$	0·81	25	0·15

Malonamide	150	25	36	0·05	$\frac{9}{13}$	$\frac{7}{14}$	0·72	$\frac{8}{10}$	$\frac{11}{14}$	0·98	$\frac{8}{14}$	$\frac{3}{14}$	0·38	$\frac{10}{7}$	$\frac{3}{14}$	0·15	44	0·3
Geranio	60	15	18	0·2	$\frac{10}{11}$	$\frac{6}{14}$	0·47	$\frac{16}{26}$	$\frac{8}{14}$	0·93	$\frac{9}{15}$	$\frac{6}{14}$	0·71	$\frac{8}{14}$	$\frac{10}{14}$	1·25	16	0·2
Citral	150	25	13	0·05	$\frac{10}{11}$	$\frac{1}{14}$	0·08	$\frac{9}{18}$	$\frac{-6}{17}$	*−0·70*	$\frac{20}{9}$	$\frac{5}{14}$	0·16	$\frac{16}{28}$	$\frac{0}{14}$	0	*111*	0·01
Geranic acid	100	20	0	—	$\frac{9}{14}$	$\frac{4}{14}$	0·45	$\frac{8}{14}$	$\frac{8}{14}$	1·00	$\frac{22}{6}$	$\frac{9}{14}$	0·17	$\frac{15}{6}$	$\frac{15}{14}$	0 43	49	0·2
Hildebrandt acid	100	10	3	—	$\frac{8}{12}$	$\frac{-6}{15}$	*−0·60*	$\frac{10}{7}$	$\frac{11}{14}$	0·55	$\frac{10}{14}$	$\frac{13}{14}$	1·30	$\frac{10}{22}$	$\frac{9}{14}$	1·41	33	0·4
Hildebrandt acid	—	25	10	0·3	$\frac{14}{7}$	$\frac{20}{14}$	0·71	$\frac{9}{11}$	$\frac{11}{14}$	0·96	$\frac{7}{11}$	$\frac{-5}{14}$	*−0·56*	$\frac{17}{32}$	$\frac{2}{14}$	0·27	66	0·2
Acrolein	1	0·25	25	0·05	$\frac{19}{21}$	$\frac{-3}{14}$	*−0·23*	$\frac{10}{28}$	$\frac{4}{14}$	0·80	$\frac{9}{11}$	$\frac{11}{14}$	0·96	$\frac{12}{21}$	$\frac{-1}{14}$	*−0·23*	67	0·1
Acrylic acid	60	15	19	0·1	$\frac{7}{17}$	$\frac{2}{14}$	0·35	$\frac{9}{14}$	$\frac{12}{14}$	1·33	$\frac{8}{7}$	$\frac{9}{14}$	0·56	$\frac{8}{6}$	$\frac{12}{14}$	0·64	28	0·2
Crotonaldehyde	6	0·5	19	0·01	$\frac{13}{19}$	$\frac{2}{14}$	0·21	$\frac{14}{14}$	$\frac{0}{14}$	0	$\frac{7}{18}$	$\frac{7}{14}$	1·28	$\frac{9}{23}$	$\frac{3}{14}$	0·54	49	0·2
Crotonic acid	120	25	5	—	$\frac{7}{18}$	$\frac{6}{14}$	1·10	$\frac{9}{14}$	$\frac{9}{10}$	1·40	$\frac{12}{10}$	$\frac{10}{14}$	0·59	$\frac{10}{14}$	$\frac{6}{14}$	0·60	8	0·5
Maleic acid	60	15	3	0·8	$\frac{9}{24}$	$\frac{4}{14}$	0·76	$\frac{7}{18}$	$\frac{9}{14}$	1·47	$\frac{16}{10}$	$\frac{9}{14}$	0·40	$\frac{10}{10}$	$\frac{9}{14}$	0·64	18	0·2
Furfuryl alcohol	40	2·5	3	—	$\frac{12}{20}$	$\frac{6}{14}$	0·71	$\frac{9}{11}$	$\frac{12}{14}$	1·05	$\frac{7}{9}$	$\frac{14}{14}$	1·28	$\frac{24}{12}$	$\frac{15}{14}$	0·54	10	0·5
Furfuraldehyde	10	2·5	15	0·01	$\frac{10}{21}$	$\frac{-3}{14}$	*−0·44*	$\frac{7}{10}$	$\frac{11}{14}$	1·12	$\frac{8}{10}$	$\frac{9}{14}$	0·80	$\frac{10}{7}$	$\frac{9}{14}$	0·45	52	0·2
Furfuramide	30	7·5	14	0·2	$\frac{14}{26}$	$\frac{3}{14}$	0·40	$\frac{29}{20}$	$\frac{6}{14}$	0·30	$\frac{18}{21}$	$\frac{7}{14}$	0·58	$\frac{9}{9}$	$\frac{11}{14}$	0·71	50	0·02
Furoic acid	25	5	0	—	$\frac{10}{24}$	$\frac{7}{14}$	1·20	$\frac{15}{12}$	$\frac{13}{14}$	0·74	$\frac{15}{21}$	$\frac{8}{14}$	0·80	$\frac{13}{17}$	$\frac{6}{14}$	0·63	16	0·2
Furfuracrylic acid	20	5	44	0·01	$\frac{14}{24}$	$\frac{4}{14}$	0·49	$\frac{9}{10}$	$\frac{7}{14}$	0·55	$\frac{9}{14}$	$\frac{9}{14}$	1·00	$\frac{9}{10}$	$\frac{5}{14}$	0·40	39	0·5
Cuminal	60	5	0	—	$\frac{10}{16}$	$\frac{10}{14}$	1·14	$\frac{11}{32}$	$\frac{0}{14}$	0	$\frac{10}{19}$	$\frac{6}{14}$	0·82	$\frac{14}{24}$	$\frac{2}{14}$	0·24	45	0·2
Phloroglucin-aldehyde	80	5	0	—	$\frac{11}{24}$	$\frac{0}{14}$	0	$\frac{9}{10}$	$\frac{11}{14}$	0·87	$\frac{12}{10}$	$\frac{0}{14}$	0	$\frac{11}{14}$	$\frac{6}{14}$	0·55	65	0·05

In this case the inhibition is insignificant. The probability (P) that the reduction of growth during treatment might have arisen by chance is about 0·6 ($t=0·50$).

Heptaldehyde and oxidation products

The effect of daily administration of 50 mg. heptaldehyde on primary carcinomata is significant, but dosage at this level causes the mice to become ill and frequently they die with one week's treatment. Heptoic acid, the simplest oxidation product of heptaldehyde, was ineffective against both grafted and spontaneous tumours. Although heptaldehyde would probably be largely converted into heptyl alcohol and heptoic acid in the body [Parnas, 1910] a certain proportion might be expected to combine with free amino groups and suffer ω-oxidation and subsequent further oxidation to pimelic acid. (This would be analogous to the metabolism of citral, cf. below.) In order to test this, the urine of rats and rabbits which were fed with heptaldehyde was examined for pimelic and other acids but none was isolated. It is, therefore, probable that heptaldehyde is completely oxidized in the body. Pimelic acid and its possible β-oxidation products, glutaric acid and malonic acid, are probable intermediates. Of these dicarboxylic acids pimelic acid had a slight effect, while malonic acid and sebacic acid had definite inhibitory actions, which suggests that the effect of heptaldehyde may not be entirely due to the toxic action of the aldehyde group. Of this group of compounds malonic acid was much more effective than heptaldehyde. Ethyl malonate and malonamide were less active. Malonic acid is known to inhibit tissue respiration *in vitro* but the respiration of slow-growing tumours in mice treated with malonic acid was not lower than that of normal tumours.

Certain dicarboxylic acid derivatives had inhibitory action. Sebacic acid dihydrazide (for which I am indebted to Major G. A. H. Buttle) was less active than the parent acid but still gave a significant effect. Adipamide was a little better than adipic acid but of this group only mucic acid (tetrahydroxyadipic acid) had a significant effect.

It is of interest that certain polymethylene diamines such as cadaverine and tetradecamethylene diamine also have an inhibitory action of the same order as the active dicarboxylic acid. Thus certain polymethylene compounds with either acidic or basic end groups are able to inhibit the growth of tumours.

Unsaturated aldehydes and acids

Citral with two double bonds and two methyl groups has more inhibitory action on tumour growth than heptaldehyde. No compounds related to citral have been found as effective as citral itself. When citral is fed to dogs or rabbits it suffers ω-oxidation to give "Hildebrandt acid" [Hildebrandt, 1901] or 2:6-dimethyl-Δ-2:6-octadiene-2:8-dicarboxylic acid. This acid was prepared, therefore, from the urine of rabbits which had been fed citral, and was found to be much less active than citral as was geranic acid (the simple monocarboxylic acid corresponding to citral). The alcohol geraniol was devoid of inhibitory action. Both geraniol and geranic acid are converted into Hildebrandt acid [Kuhn & Livada, 1933] in the body. Comparison of the effects of these terpenoid compounds suggests that the aldehyde group is necessary for maximal activity, and that it is not the known metabolic product which is responsible for the inhibitory effect.

Acrolein, crotonaldehyde and furfuraldehyde inhibit the growth of chick fibroblasts in tissue culture [Willmer & Wallersteiner, 1939]. The two simple unsaturated aldehydes acrolein and crotonaldehyde were only effective against grafted tumours. Acrylic acid and crotonic acid were almost without action,

indicating that the toxic aldehyde group is responsible for the activity of the corresponding aldehydes. Maleic acid, the simplest unsaturated dicarboxylic acid which could be expected to have any effect, had no significant inhibitory action.

Cyclic compounds

Furfuraldehyde, like acrolein and crotonaldehyde, had little effect on spontaneous tumours but had a definite action on grafted sarcomata. Furfuramide, the ammonia derivative of furfuraldehyde, was only active on carcinomata and furfuryl alcohol and furoic acid were ineffective against both types of tumour. Furfuraldehyde differs from other aldehydes in its metabolism, being excreted as furfuracrylic acid. Furfuracrylic acid (synthesized by Perkin's reaction on furfuraldehyde) had about the same effect as furfuraldehyde so that it is possible that the action of the aldehyde might be due to the metabolic product.

Cuminal, an aromatic aldehyde related to the terpenes, had no significant inhibitory action on either carcinomata or grafted sarcomata. Phloroglucinaldehyde, which was the most promising aromatic aldehyde previously tested [Boyland & Mawson, 1938], caused significant inhibition of carcinoma growth.

Discussion

Consideration of the possible fate of heptaldehyde in the body suggested that ω-oxidation might occur and give pimelic acid which might in its turn be oxidized to glutaric and malonic acids. All these acids have some inhibitory action and that of malonic acid seems to be greater than that of heptaldehyde itself. This indicates that certain dicarboxylic acids, like some diamines, have inhibitory action and opens up a new field for investigation.

Citral is much more effective than heptaldehyde but none of the citral derivatives which have been tested has the activity of citral; other unsaturated aldehydes which were examined were very toxic and not very active.

Summary

1. An attempt has been made to express quantitatively and statistically the effect of substances which inhibit tumour growth.

2. Heptaldehyde given to mice has some inhibitory action on the growth of spontaneous carcinomata and grafted sarcomata. A similar effect is produced by dicarboxylic acids (particularly malonic acid) which are possible metabolic products of heptaldehyde.

3. Citral has more inhibitory action on tumour growth than heptaldehyde but the monocarboxylic and dicarboxylic acids derived from citral have only slight effect.

I should like to express my thanks to Dr J. W. Trevan to whom I am indebted for help in the statistical treatment of the data, and to the British Empire Cancer Campaign for grants which have supported this research.

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143. MICRO-DETERMINATION OF COPPER IN BIOLOGICAL MATERIAL

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(*Received 13 July 1940*)

In work on the physiology of Cu [Eden & Green, 1939; Eden, 1939; 1940, 1, 2,], involving unusually large numbers of determinations on blood, tissues, urine, faeces and animal foods, the Tompsett modification [1934, 1] of the method of McFarlane [1932], itself based on the diethyldithiocarbamate colour reaction of Callan & Henderson [1929], was at first explored. Apart from the somewhat laborious ashing or tissue extraction procedures, the technique for subsequent deionization of iron was not found universally satisfactory. Although results of the correct order of magnitude were generally obtained, and might therefore not be suspected at sight, the general drift was definitely high and the slightest imperfection in removal of iron from the sphere of reaction seriously affected percentage accuracy as real magnitudes of Cu diminished, so rendering the process uncertain for metabolism work in which an accurate balance sheet was required.

In the case of blood mere increase in the recommended quantity of deionizing pyrophosphate effected improvement, but since the same difficulty has been encountered by van Niekerk [1937] and by others (personal communications), and since the technique finally adopted at this laboratory, and subsequently used for several thousand determinations, not only guards against imperfect deionization of iron but also embodies simplifications of manipulation, enhanced speed, increased sensitivity and more compact apparatus, its recording should prove of service. The speed and simplicity are gained by combining a rapid wet combustion method with colour development in strongly alkaline medium, and subsequent shaking out with amyl alcohol, in the combustion tube itself. Sensitivity is increased by photometric measurement on reduced extraction volumes in micro-tubes providing adequate stratum thickness. A single operator can complete 30 determinations a day and, if necessary, real magnitudes as low as 0·3 μg. Cu, the quantity present in 0·5 ml. normal blood, can be read to the second significant figure, thus extending the method to work on small laboratory animals.

With regard to obviation of interference by formation of the coloured diethyldithiocarbamate compound of iron before extracting the corresponding compound of Cu, it is not always realized that deionization of iron with pyrophosphate [Warburg, 1927] is enormously enhanced by ensuring adequate concentration of ammonium salts in solution. The completely deionized compound is an iron ammonium pyrophosphate and not iron pyrophosphate itself; so that, provided that the concentrations of ammonium ions and pyrophosphate ions are made sufficiently high, the final colour development of the Cu compound can be conducted at any level of alkalinity at all. The tedious bringing to "slight alkalinity to litmus" and subsequent "heating to 80° for 15 min. and cooling" of MacFarlane and Tompsett is thus eliminated altogether. The same deionization behaviour holds when citrate is used to prevent precipitation of calcium phosphate. Ferric

ammonium citrate is completely unionized in presence of sufficient ammonium and citrate ions, so that it is unnecessary to use pyrophosphate for deionization when citrate is already present—a point apparently not realized either by Tompsett [1934, 2] or van Niekerk. Furthermore, manganese is deionized as completely as iron, both by citrate and by pyrophosphate in presence of sufficient excess of ammonium salts. The advantage of pyrophosphate over citrate is largely an aesthetic one; a colourless solution is obtained so that any colour subsequently formed with diethyldithiocarbamate is due to Cu and the completeness of its removal with amyl alcohol is apparent to the naked eye. Extraction from the slightly coloured citrate medium is equally complete although less obvious, but any alien colour, arising from possibly imperfect combustion, is masked.

With regard to colour development both McFarlane and Tompsett use 0·5 mg. 2% aqueous sodium diethyldithiocarbamate. Under the conditions described for deionization of the iron from 5 ml. blood with 5 ml. pyrophosphate interference can readily occur if the aqueous reagent is simply added without attention to "local excess factors" which dilute the deionizing pyrophosphate in regions of high concentration of diethyldithiocarbamate. van Niekerk evades the potential error by using 0·5 ml. of 0·5% concentration of reagent instead of 2·0%, but this reduction of total diethyldithiocarbamate is unnecessary if the concentrations of pyrophosphate and ammonium ions are raised and the reagent is added dropwise from a burette with constant shaking. In the present method dropwise addition of 2 ml. 0·5% reagent replaces uncontrolled addition of 0·5 ml. 2%.

With regard to destruction of organic matter McFarlane and van Niekerk used dry incineration. Tompsett either adopted extraction with trichloroacetic acid or an efficient but cumbersome wet combustion, involving evaporation to dryness with nitric acid on a water bath and subsequent completion of digestion with sulphuric and perchloric acids. In the technique adopted by us the main oxidation is arranged to occur at the expense of nitric and perchloric acids, sulphuric acid being merely used, in amount dictated by the final volume desired, to raise the temperature in the last stages of combustion and drive off most of the residual perchloric acid. The compact variation now described for destruction of anything up to 2 g. "dry matter" is clean, safe, rapid and of wide general application. The small combustion stand[1] with six guarded micro-burners allows accurate control of digestion and is also suitable for various micro-distillations.

Combustion. Quantities of the order of 5 ml. blood, 1–5 g. wet tissues or 1 g. dry food are introduced into an 8 × 1 in. pyrex test tube scratched at approximately 25 ml.; 1 ml. A.R. H_2SO_4, 3 ml. Cu-free $HClO_4$ and two glass beads are added, and the mixture gently heated over the lowered flame of a micro-burner. With blood a little initial frothing occurs but this is easily controlled. Water is driven off by gentle boiling and heating is continued until rings of darkened mixture begin to ascend the tube. The flame is then turned out, the tube allowed to cool for about a minute and 2–3 ml. A.R. HNO_3 carefully added from a teat pipette. If previous heating has been carried to the correct stage of "ascent of darkened rings" there is no undue frothing and controlled heating may be resumed at once. The dark brown liquid gradually lightens in colour, passing through orange to faint greenish-yellow or even colourless. Stronger heating is continued a few minutes past the pale final stage to drive off most of the residual $HClO_4$ and leave a final volume of about 1 ml., i.e. the quantity of H_2SO_4 originally added. The stages of combustion at the various boiling points of the three acids

[1] Made to order by Gallenkamp Ltd. and illustrated in catalogue, 11th edition, p. 1216.

go quietly and are readily distinguishable by the character of ebullition. If the HNO_3 is driven off too fast the liquid may change from orange to black as the boiling point rises to that of $HClO_4$, in which case the flame is turned down and a few more drops of HNO_3 added. It is essential to effect complete combustion of organic matter so as to obviate any alien tint in the subsequent alkalinization with NH_3 and extraction with amyl alcohol.

A complete single combustion of quantities of material of the order mentioned takes 30–45 min., but, since 6 tubes (1 stand) or 12 tubes (2 stands) can be supervised simultaneously, the average working time per determination can be reduced to 5 min.

For urine the combustion procedure has to be slightly modified because of the larger volume required to obtain sufficient Cu for subsequent determination. 50 ml. urine are measured into a 100 ml. pyrex Kjeldahl flask (of suitable neck diameter if the same clips of the micro-stand are to be used) and about 3 ml. HNO_3 added, with two glass beads to prevent bumping. Heating is carefully controlled to bring through the boiling point without undue frothing, and smooth boiling is continued over a low flame until the volume is reduced to about 2 ml. The flame is then turned out and, after cooling, the combustion procedure with 1 ml. H_2SO_4, 3 ml. $HClO_4$ and a few ml. HNO_3, follows exactly as above. In the final stages of evaporation of urine the thick mass in the combustion flask begins to splutter and a small harmless conflagration may occur with burning away of carbon. This makes no difference to the subsequent procedure. A considerable quantity of salts separates out in the final stages of digestion and after completion a few drops of water are added to the flask while still warm, to prevent solidification. The contents are then washed with about 10 ml. water into the usual 8×1 in. pyrex tube scratched at 25 ml.

Deionization. As a general procedure with materials of uncertain Ca and P content deionization of Fe follows with ammonium citrate, since this also prevents precipitation of calcium phosphate on subsequent alkalinization. The combustion residue in the tube is diluted with 5–10 ml. water and 2 ml. 50% ammonium citrate added, followed by 5 ml. ammonia sp. gr. 0·880. Of this ammonia about 3 ml. go to neutralization of the H_2SO_4 and formation of the ammonium sulphate desired to promote deionization, and the residual 2 ml. to rendering strongly alkaline. Water is then added to the 25 ml. mark. The solution is yellowish but clear, although slight opalescence does not matter. Unnecessarily long standing at this stage may cause slight opalescence if the amount of calcium phosphate present is high.

For material relatively low in Ca and P but high in Fe, such as blood or soft tissues, the pyrophosphate method of deionization is equally good, and preferable for reasons mentioned above. In place of 2 ml. 50% ammonium citrate 10 ml. 4% hydrated sodium pyrophosphate are used (i.e. large excess, twice the quantity of the McFarlane or Tompsett procedures), followed by 5 ml. ammonia sp. gr. 0·880 and making up to about 25 ml. as before. For some tissues comparatively rich in Ca and P, and for faeces, the colourless pyrophosphate method of deionization can also be used provided that the precipitate formed on alkalinization is boiled to render it granular. With urine, however, the citrate method should always be used since the fine precipitate first formed does not readily granulate and Cu may be occluded.

Colour development and extraction. 2 ml. of 0·5% recently filtered sodium diethyldithiocarbamate[1] are now added from a burette with constant shaking,

[1] Diluted from a stock 2% aqueous solution stored in the dark. Any deposit formed is filtered off before diluting aliquots for daily use.

followed by exactly 5 ml. amyl alcohol. The tube is then closed with a clean rubber bung and vigorously shaken for ½ min., a period sufficient to ensure extraction of the yellow copper compound. In the presence of the electrolytes in solution the amyl alcohol rapidly separates to the top. Sufficient is taken off with a teat pipette for colour measurement, and filtered through acid-extracted filter paper, or alternatively centrifuged, to remove suspended water. It is important to use extracted papers since amyl alcohol may acquire a faint yellowish tint in passing through some grades of ordinary filter paper. The quantity of amyl alcohol pipetted off is of course immaterial since the Cu content is measured by the intensity of colour conferred on the precisely measured 5 ml. amyl alcohol added to the approximately constant extraction volume of 25 ml. Extraction is practically complete with a single shaking and the solubility of amyl alcohol in water is slight, but both factors are allowed for by treating the standard Cu solutions in exactly the same way. A quantity of 3 ml. to 3·5 ml. filtrate is obtained without undue trouble, enough to fill the micro-cups of a Klett biocolorimeter and much more than required for the 1 ml. micro-tube of a Zeiss photometer. If the latter is available the quantity of amyl alcohol used for shaking out can be reduced to intensify the colour when unusually low Cu is encountered.

Colour measurement. Comparison may be made with narrow Nessler tubes, in colorimeter cups or, best of all, with a Zeiss "step photometer" measuring light extinction within a selected spectral range. In each case comparative standards are required, although in the case of the photometer they are only needed once in order to plot permanent graphs of extinction coefficients with different light filters. The stock Cu solution is prepared by dissolving 0·3928 g. $CuSO_4$, $5H_2O$ in 1 l. of water containing a drop of H_2SO_4, giving 0·1 mg. Cu per ml. This is diluted 1 in 10 as required for preparation of standards. Convenient matching ranges are made by transferring 0·5 ml., 1 ml. and 2 ml. of the diluted stock, corresponding to 0·005, 0·01 and 0·02 mg. Cu, to the 8×1 in. pyrex tubes, adding the same quantities of citrate or pyrophosphate and NH_3 as after combustion of unknowns, and extracting with 5 ml. amyl alcohol after dilution to 25 ml. and addition of carbamate reagent. All reagents[1] should of course be tested for freedom from Cu, and the magnitude of the "total blank" must be accurately determined and allowed for.

For photometric measurement a "total blank" is obtained by carrying through the whole process, including the combustion but omitting the organic matter, and using the amyl alcohol layer so obtained for the compensating cell of the instrument. In this case any traces of Cu in the reagents are automatically allowed for and the difference in extinction coefficients on the two sides corresponds exactly to the Cu in the material examined. Micro-tubes of 1 ml. capacity are used and since these give a stratum thickness of 50 mm. great sensitivity is obtained.

The yellow colour of copper diethyldithiocarbamate absorbs light over the whole visible spectrum, least in the red and most in the violet. Filters S 53, S 50 and S 47, corresponding to mean wave lengths of 528, 488 and 458 mμ are the most useful. S 53 may be used for quantities of Cu from 0·02 mg. down to 0·005 or even 0·002 mg., in the 5 ml. amyl alcohol used for extraction. Unexpectedly high ranges are dealt with by dilution with amyl alcohol. For low ranges S 50 and S 47 are preferable as giving greater extinction coefficients. In general the filter selected should show a conveniently readable coefficient of 0·1–0·5, corresponding to absorption of one-fifth to two-thirds of the total light, higher

[1] Copper-free $HClO_4$ is supplied by Hopkins and Williams Ltd.

and lower readings being inaccurate. If the Cu present gives a lower extinction coefficient than 0·12 the next higher filter is switched in. With a coefficient of 0·20, representing about one-third light absorption, the maximum reading error is within ±0·1 and by taking the mean of five rapid readings the error can easily be reduced to ±1%. The quantity of Cu is directly proportional to the extinction coefficient and graphs for varying concentrations are therefore straight lines passing through the origin. Each analyst should prepare his own graphs from his own standards, so equilibrating the personal equation of his total technique once and for all.

Table 1 shows the general relationships for filters S53, S50 and S47:

Table 1. *Extinction coefficients for Cu with various filters in 50 mm. stratum thickness*

mg. Cu present	S 53	S 50	S 47
0·0010	—	0·050	0·132
0·0020	0·067	0·143	0·302
0·0050	0·174	0·356	0·708
0·0075	0·259	0·559	1·101
0·0100	0·348	0·734	—
0·0150	0·531	1·089	—
0·0200	0·683	—	—

It will be noted that within the recommended reading range and the limits of error the values lie on straight lines, and that even for so small a magnitude as 0·001 mg. Cu the S47 filter gives the satisfactory reading of 0·132.

If a photometer is not available the ordinary Klett biocolorimeter, with micro-cups giving a convenient reading depth with the available 3–3·5 ml. amyl alcohol filtrate, is satisfactory, and quantities down to 0·001 mg. Cu per 5 ml. can be read with fair accuracy, using a standard sufficiently close to the unknown. The Cu colour is not strictly monochromatic and it is generally necessary to make up at least three standards (0·005, 0·01 and 0·02 mg. Cu) simultaneously with the unknown and set the most suitable one between 15 and 30 mm. With a standard of 0·01 mg. set at 20 mm. the reading with 0·02 mg. is about 10·7 mm. instead of the theoretical 10 mm., giving a result about 14% too low; similarly a real quantity of 0·003 mg. Cu read against an 0·01 standard set at 10 mm. reads about 28·2 mm., giving a result about 15% too high. In general, if it is desired to keep the error within ±5% it is necessary to discard readings farther apart than 15 and 30 mm. for a standard set at 20 mm. To attain the ±1% accuracy readily obtained with the photometer and permanent graphs, it is necessary to prepare a closer range of colorimeter standards and read within a few mm. of the unknown. Standards should be made up at the same time as the unknowns to obviate light fading and evaporation changes. Although the colour of the Cu compound in amyl alcohol is fairly stable and no appreciable change occurs in diffuse light in several hours, exposure to strong sunlight in presence of air effects slow fading.

For very low concentrations of Cu, such as 0·001 mg. in 5 ml. amyl alcohol, narrow Nessler tubes are preferable to the colorimeter since greater depth is available for colour differentiation. Simple tubes, such that a 2 ml. quantity of fluid provides a depth of about 16 cm., are convenient.

Accuracy of method. Numerous controls have been carried out with known quantities of Cu and Fe added to Cu-free organic matter before combustion, and by recovery of known quantities of Cu added to blood, liver tissue, urine and faeces. With real magnitudes of 0·01 mg. Cu or more there is no difficulty in

keeping the total error within ±2%, i.e. returning 0·0098–0·0102 mg., especially if the photometric finish is employed. As real magnitudes diminish the error increases and for quantities about 0·001 mg. an error of ±10% must be regarded as permissible with the procedure already described. By further refining the technique, however, reducing the dilution volume of combustion residue and the amount of amyl alcohol used for extraction, the higher accuracy can be restored and still smaller quantities of Cu satisfactorily estimated.

Further refinement of technique. It is obvious that the limiting factor in the sensitivity of the method is simply the intensity of the colour, which in turn depends upon the extent to which the volume of extracting amyl alcohol can be kept down, and the reading limits of the instrument. With the Zeiss photometer the smallest cell obtainable requires 1 ml. and to cover marginal losses this necessitates the use of a minimum of 1·5 ml. amyl alcohol for extraction. Taking 0·10–0·15 as a satisfactory coefficient for accurate reading with an S 47 light filter, the satisfactorily determinable quantity of copper comes down to 0·2–0·3 μg. Ability to determine such small magnitudes then brings the method within the scope of work in which other factors impose stricter limits upon the amount of material available, e.g. experiments upon the white rat from which 0·5 ml. blood is readily obtainable, although 5 ml. is not, or upon single plants in which the morphological distribution of Cu may be wanted.

The difficulties are purely mechanical, involving greater attention to levels of contamination immaterial with 3 μg. Cu but disastrous with 0·3 μg. For this reason the whole scale of operations is reduced, centrifugal separation of the aqueous haze in the amyl alcohol layer takes the place of filtration, and one or more controls with known traces of Cu are inserted in every series of combustions. The variation, as applicable to blood, is as follows.

0·5 ml. blood is measured into a specially cleansed 8 × 1 in. pyrex tube, followed by 0·3 ml. each of H_2SO_4 and $HClO_4$. At the same time a control tube omitting the blood is set up for use as blank in the compensating cell of the photometer, with a second control tube containing 0·3 μg. Cu from highly diluted stock standard. Digestion proceeds in the normal manner, about 0·3 ml. HNO_3 being used for each tube. After combustion is apparently complete a further two drops each of $HClO_4$ and HNO_3 are added and heating is repeated to ensure removal of the last trace of organic matter. If this is not done a faint yellowish colour may appear on subsequent alkalinizing, barely visible to the eye but nevertheless extractable by amyl alcohol and registering as Cu. To the final digestion residues, consisting mainly of the 0·3 ml. H_2SO_4 originally used, are added 2 ml. 4% sodium pyrophosphate (or 2 ml. 10% ammonium citrate if the material is high in Ca and P), followed by 2 ml. ammonia sp. gr. 0·880 and eight drops (0·4 ml.) of recently filtered 0·5% sodium diethyldithiocarbamate. The contents are then washed into 15 ml. centrifuge tubes with two successive portions of about 1 ml. water, bringing the total volumes to approximately 7 ml. in each case. Exactly 1·5 ml. amyl alcohol are added, the centrifuge tubes closed with clean well-fitting rubber bungs, vigorously shaken for ½ min., centrifuged at about 2500 r.p.m. for 10 min., removed, gently agitated to ensure coalescence of the main bulk of the amyl alcohol with that newly risen and centrifuged a further 5 min. Enough of the amyl alcohol layer is then carefully removed, with a teat pipette drawn to capillary point, to fill the micro-cell of the photometer, the "blank" going into the compensating cell. Filter S 47 is used for readings, calculations being made from charts previously constructed by the same analyst. The control tube containing the known 0·3 μg. suffices to check the accuracy of manipulation throughout.

Although scrupulous care is required for handling such minute real magnitudes there is no deviation from simplicity and, as shown in Table 2, results are nearly as good with 0·5 ml. as with 5 ml. of blood.

Table 2. *Comparison of results obtained on 0·5 and 5 ml. portions of blood using the technique described.*

Filter S 47 employed

No.	Quantity blood ml.	Extinction coefficient	Absolute quantity of Cu found mg.	Cu per 100 ml. mg.
1	5	0·460	0·0031	0·062
2	5	0·455	0·0031	0·062
3	5	0·450	0·0031	0·062
4	0·5	0·160	0·00032	0·064
5	0·5	0·155	0·00032	0·064
6	0·5	0·150	0·00031	0·062
7	0·5	0·170	0·00035	0·070

Summary

The diethyldithiocarbamate method for estimation of Cu in biological material is improved in simplicity, speed, accuracy, and sensitivity. Rapid wet digestion, with a combination of sulphuric, perchloric and nitric acids in 8×1 in. pyrex test tubes, is employed for destruction of organic matter, followed by deionization of iron with citrate or pyrophosphate in strongly alkaline medium. Subsequent colour formation and extraction with amyl alcohol are effected in the combustion tube itself. With quantities such as 5 ml. blood containing 3 μg Cu, the final reading is either colorimetric or photometric, but with limited material from small laboratory animals, involving determination of real amounts of Cu as low as 0·3 μg., photometric finish with a low wave-length light filter is essential. About 30 determinations per day can be completed by a single analyst.

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144. A MICRO-KJELDAHL APPARATUS

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(*Received 25 July 1940*)

THE apparatus described below is suitable for the estimation of amounts of N as small as 50 μg. In order to avoid any great increase in the volume of the reacting solutions during the steam distillation the usual apparatus was modified as follows.

The distillation apparatus (see Fig. 1) consists of two communicating bulbs. The upper one (*A*) is fitted with a trap which is continuous with the distillation tube, which in turn is surrounded by a condenser. The lower bulb (*B*) has a

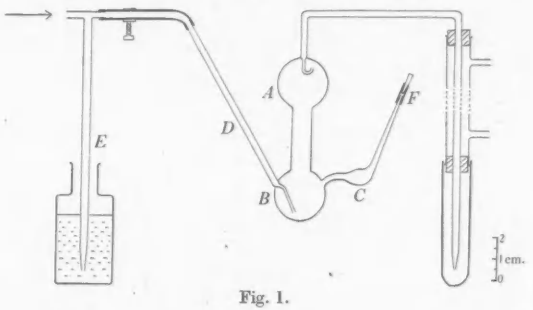

Fig. 1.

slightly widened bent tube (*C*) on one side to receive one of the solutions, and a small tube (*D*) on the other side to serve as a connexion with the vapour source. To assure continuous regular boiling a vapour trap (*E*) is inserted. The whole apparatus, including the condenser, is made in one piece of pyrex glass and can be tilted round a horizontal axis perpendicular to the plane *A*, *B*, *C*, *D*.

The incineration of the substance, containing some 50–150 μg. N, is carried out by means of 0·1 ml. H_2SO_4, with $CuSO_4$ as a catalyst and finally 1 drop of H_2O_2. The colourless clear liquid is transferred into the widened part (*C*) of the bent tube of the lower bulb by means of a capillary pipette and the tube is then closed with a glass stopper (*F*) inserted into a rubber tube. Into the lower bulb 1 ml. 33% KOH has been introduced previously through (*D*) with a drop of liquid paraffin to avoid foaming. The lower bulb is then connected to a steam generator by rubber tubing. When bubbles begin to pass through the KOH solution the apparatus is tilted so that the solution of incinerated substances runs into the bulb (*B*). During the distillation the bulb (*C*) should be cooled by the external application of a wad of cotton-wool soaked in cold water. When a small amount of water has condensed in (*C*) the apparatus is again tilted so that this water runs back into *B*; this washes the remains of the solution out of (*C*). It is advisable to heat the upper part of the apparatus with a free flame in order to prevent condensation. The distillation should be continued for 6 min., the last 3 min. serving

to wash out the tube. The distillate is collected in a known amount of $N/100$ HCl and after adjustment to a definite volume, e.g. 6 ml., the excess of HCl is determined iodometrically in the usual manner. The accuracy of the method is $\pm 3\%$, using 0·2–0·3 mg. of substances containing about 10–30% N.

Example: 1·24 mg. cytosine were dissolved in 1 ml. water. 0·192 ml. of the solution containing 0·238 mg. cytosine was incinerated and the N content estimated.

N content calculated: 89μg.=37·6%. Found: (1) 92·5μg.=38·8%; (2) 88·2μg.=37·0%.

I wish to express my indebtedness to Mr Frank Goulden for constructing the first model of this apparatus.

145. THE QUANTITATIVE BASIS OF PECTIN JELLY FORMATION IN RELATION TO *p*H CONDITIONS

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(*Received 13 March 1940*)

Introductory

Various factors which govern the jellying properties of fruit pectins have been studied and described by Hinton [1939]. In the present paper it is proposed to devote attention to factors which can be varied at will, such as the effects of the concentrations of pectin and of other soluble substances on the strength of the jelly; the effect of the group of conditions which may conveniently be summarized under the term *p*H; and the effects of such a minor modification of the pectin molecule as the de-esterification resulting from the action of pectase or of alkali.

It will be shown that pectins may be studied to a considerable extent from an ordinary physico-chemical standpoint, taking into account in particular the properties of solubility and of electrolytic dissociation. Hinton [1939] suggested that they must be regarded as complex mixtures of carboxylic acids of variable composition. It is impossible at present to separate the molecular components; hence the properties cannot be dealt with stoichiometrically. They can, however, be treated in a statistical sense, and on this basis it is possible to establish much useful quantitative knowledge and to correlate this with the jellying behaviour.

The superficial connexion between *p*H and the strength exhibited by a pectin jelly has been familiar for some time [Hinton, 1939, p. 15]. As the *p*H of a jellying mixture is raised the strength of the jelly it forms diminishes (other conditions, such as total solids concentration, remaining unaltered). If the *p*H is raised sufficiently, the mixture fails to form a jelly; on the other hand, if acid is added the jelly increases in strength down to a *p*H at which the strength reaches a maximum.

Pectins in dilute solution may be expected to behave like other organic acids. Dissociation of the acid groups must occur, and the degree to which this occurs will be related to the dissociation constants of the successively dissociating acid groups. The physical state of the pectin—i.e. its degree of coherence or of dispersion as a hydrosol—is determined by the potential difference between the pectin and the solvent; this potential difference arises from the dissociation of the pectin or of its salts. If the potential is discharged by suppression of the dissociation, the coagulating tendency of the molecules is no longer hindered. Jellying may be regarded as a form of coagulation in which the coagulated particles form a continuous network [Hardy, 1900]. Hence the conditions for the formation of pectin jellies must embrace the dissociation behaviour of the pectin and of its salts.

The acid groups of the pectin, so long as they are entirely in the free condition, may be expected to be dissociated to a minor degree only; hence the

pectin is very little ionized, and coagulation or jellying can readily occur. When alkali is added in partial neutralization, the number of dissociated groups increases because of salt formation, and coagulation or jellying is inhibited. This mechanism therefore is capable of explaining why, as the *p*H rises, the jelly strength diminishes and ultimately disappears completely.

When acid (e.g. HCl) is added to a pectin already partly neutralized in this way, the process is reversed; the dissociation of the acid groups diminishes and jellying can again take place. The net effect of the addition of the alkali and the acid is virtually the addition of a small amount of a more or less inert salt (e.g. NaCl).

Neutralization data and dissociation "constants" of pectins

The dissociation of the acid groups was studied by means of partial neutralizations of pectins from which the metallic bases had been first eliminated. For the preparation of these pectins, a considerable excess of HCl was added to a dilute solution of the pectin, followed after a short time by sufficient acetone to precipitate the latter in the free acid state. One litre of 0·5 % pectin solution required 30–40 ml. of conc. HCl and 700 ml. acetone. The precipitated pectin was filtered off, washed with 70 % acetone, and put back into 2 l. 70 % acetone, in which it was allowed to soak for 2 or 3 days. The filtration and soaking were repeated 4 or 5 times, until a portion of the filtrate, after removal of the acetone, gave no opalescence with $AgNO_3$. The pectin was finally washed with 98 % acetone, spread out in a dish, and dried in a warm room. It was dissolved to a paste by mixing with pure sucrose (10 g.) and thymol (0·07 g.) and then gradually adding water while stirring so as to give a 4 or 5 % solution of the pectin. By allowing to stand with frequent stirring, and in some cases moderate warming, a smooth paste was usually obtained in a day or two. The pastes were kept in airtight glass jars.

The free acidity, saponification value, alkalinity of ash and calcium pectate yield of the pastes were determined by the methods already described [Hinton, 1939, pp. 11, 27, 28, 33]. The pectins were sometimes free from alkaline ash; in other cases there was a trace of alkalinity, which was allowed for when calculating the results on the assumption of its being due to an initial partial neutralization of the pectin with alkali.

In determining the neutralization data, the concentration was kept constant in each series. A number of separate portions of the pectin paste were weighed out into beakers provided with stirring rods, and to each portion was added a suitable amount of water followed by dilute $NaHCO_3$ (or K_2CO_3) solution of known strength. The hydroxide was purposely not used in order to avoid the risk of local saponification. Thus a series of mixtures was obtained with increasing amounts of alkali, but with the same total weight in each. The water and alkali were well stirred in and the beakers covered and left until the following day, when the weights were finally adjusted with small additions of water. The *p*H values were determined with quinhydrone, a 0·4 % solution of cream of tartar (*p*H = 3·57) being used as a reference buffer. Within the range covered it may be taken that the CO_2 liberated from the carbonate had no significant effect.

Table 1 gives the results obtained with pectins from orange, red currant and strawberry. The de-ashing treatment was less successful with the strawberry pectin. The jellying qualities of the orange and red currant pectins were good; of the strawberry, feeble.

The partial neutralizations were effected with $NaHCO_3$.

Table 1. *Partial neutralization data and dissociation constants of pectins*

Analytical details of pectins:

	As NaOH % of calcium pectate yield			
	Free acidity	Saponification value	Alkalinity of ash	Equivalent weight
Orange	4·43	12·61	0·02	899
Red currant	4·99	12·62	0·00	802
Strawberry	3·96	14·64	0·38	922

No.	Concentration of pectin: g. per 100 g. water	g. equiv. per 1000 g. water	Degree of neutralization	*p*H	*K* Dissociation "constant" $\times 10^{-4}$
Orange:					
1	0·59	0·0066	0·004	2·85	4·0
2	0·59	0·0066	0·160	3·07	3·5
3	0·59	0·0066	0·315	3·31	3·1
4	0·59	0·0066	0·473	3·62	2·5
5	0·59	0·0066	0·627	3·97	2·0
6	1·08	0·0120	0·004	2·67	4·7
7	1·08	0·0120	0·160	2·91	4·4
8	1·08	0·0120	0·315	3·16	3·9
9	1·08	0·0120	0·473	3·48	3·3
10	1·54	0·0171	0·004	2·56	5·4
11	1·54	0·0171	0·160	2·81	5·1
12	1·54	0·0171	0·315	3·11	4·4
Red currant:					
1	0·26	0·0032	0·000	3·04	3·6
2	0·26	0·0032	0·159	3·21	3·3
3	0·26	0·0032	0·318	3·43	2·8
4	0·51	0·0064	0·000	2·83	4·5
5	0·51	0·0064	0·159	3·04	4·0
6	0·51	0·0064	0·318	3·28	3·5
7	0·51	0·0064	0·478	3·60	2·7
8	0·51	0·0064	0·636	3·93	2·2
9	0·94	0·0117	0·000	2·67	4·8
10	0·94	0·0117	0·159	2·91	4·4
11	0·94	0·0117	0·318	3·19	3·9
12	0·94	0·0117	0·478	3·52	3·1
13	1·34	0·0167	0·000	2·57	5·2
14	1·34	0·0167	0·159	2·86	4·4
15	1·34	0·0167	0·318	3·15	4·0
Strawberry:					
1	0·43	0·0047	0·088	3·01	4·1
2	0·43	0·0047	0·262	3·24	3·6
3	0·43	0·0047	0·436	3·53	2·9
4	0·43	0·0047	0·611	3·89	2·3

The last column of the table gives the values for the apparent dissociation "constant", calculated as for a monobasic acid in the usual way, on the assumption that the sodium salt was completely dissociated. Activity coefficients have been neglected.

The results for the three pectins show a general similarity. The dissociation "constant" diminishes with increasing degree of neutralization, as found by Briggs [1934] for arabic acid. This acid is closely related to the pectins [Pauli & Palmerich, 1937], but has not the complexity which pectins owe to their partial esterification. From the data of Briggs, the dissociation "constant" of arabic acid has values of the same order as those now found for pectin, and the decrease with increasing degree of neutralization is similar. Briggs ascribed this decrease to the fact that the acid groups with the greater tendency to dissociate are first

neutralized, the average dissociation constant of the remaining groups thus being lowered.

The value of K at a given degree of neutralization decreased with dilution. In the case of the red currant pectin, this dilution effect appears to diminish at the higher concentrations, as can be seen from Fig. 1. There appears to be an approach to a constant value for each degree of neutralization. Briggs found similar effects for arabic acid.

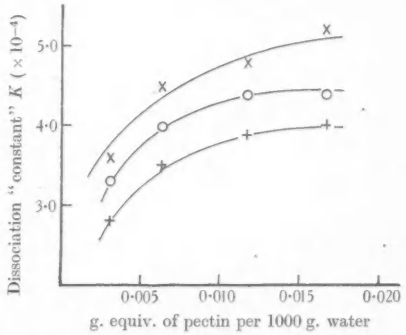

Fig. 1. Variation of dissociation "constant" with pectin concentration (red currant pectin). Degree of neutralization = 0, ×; degree of neutralization = 0·159, o; degree of neutralization = 0·318, +.

A comparison of neutralization data with Na and K as neutralizing metals was made, a second sample of red currant pectin being used (Table 2). This was of interest in connexion with differences in the jellying behaviour in presence of Na or K salts, to be discussed later. $NaHCO_3$ and K_2CO_3 solutions were used for the neutralizations.

Table 2. *Partial neutralization data with sodium and potassium as neutralizing alkalis*

Red currant pectin No. 2

Analytical details of pectin: Free acidity 5·27% (as NaOH % of calcium pectate yield)
Saponification value 13·30% (as NaOH % of calcium pectate yield)
Alkalinity of ash 0·01% (as NaOH % of calcium pectate yield)
Equivalent weight 758

Concentration of pectin in each mixture = 0·118 g. equiv. per 1000 g. water

No.	Alkali used	Degree of neutralization	*p*H	$K \times 10^{-4}$
1	No addition	0·002	2·23	3·2
2	Na	0·152	2·73	3·7
3	,,	0·271	3·00	3·9
4	,,	0·384	3·23	3·8
5	,,	0·482	3·42	3·6
6	No addition	0·002	2·24	3·1
7	K	0·137	2·70	3·6
8	,,	0·242	2·95	3·8
9	,,	0·373	3·21	3·8
10	,,	0·451	3·35	3·7

The neutralization curves are identical for the two neutralizations.

The values of K in the earlier stages of neutralization do not follow the same course as in the previous experiments, being unexpectedly low. This may possibly be due to the high concentration of pectin in this series; in the earlier stages of neutralization some aggregation may have occurred, thereby lowering the effective concentration of undissociated acid groups.

In the later stages of neutralization, after passing through a maximum, the values of K tend to become more concordant with those for the pectins previously examined.

The effect of the heat treatment of a pectin on its dissociation was examined, a second sample of orange pectin being used. A solution of the pectin was divided into two portions, one of which was gently boiled under reflux for 1 hr., cooled, and the ash-free pectin prepared by precipitation with HCl and acetone and purification as already described. The ash-free pectin was also prepared from the unheated portion of the solution in the same way. Neutralization data for both are shown in Table 3.

Table 3. *Partial neutralization data for pectin before and after boiling for 1 hr.*

Orange pectin No. 2

		Unboiled	Boiled
Analytical details of pectin	Free acidity	3·93	4·20 (as NaOH % of calcium pectate yield)
	Saponification value	13·33	13·68 (as NaOH % of calcium pectate yield)
	Alkalinity of ash	0·05	0·04 (as NaOH % of calcium pectate yield)
	Equivalent weight	1005	943

No.	Concentration of pectin g. equiv. per 1000 g. water	Degree of neutralization	pH	K $\times 10^{-4}$
Unboiled:				
1	0·0062	0·011	2·88	3·8
2	0·0062	0·177	3·09	3·6
3	0·0062	0·343	3·34	3·3
4	0·0062	0·510	3·64	2·8
5	0·0062	0·674	4·00	2·2
Boiled:				
6	0·0071	0·010	2·81	4·6
7	0·0071	0·155	3·00	4·2
8	0·0071	0·299	3·22	3·8
9	0·0071	0·445	3·47	3·3
10	0·0071	0·588	3·75	2·8

There is a slight difference between the two sets of values for K which is rather greater than can be accounted for by the different concentrations, except perhaps in the later stages of neutralization.

In jelly tests with these pectins at pH 3, the comparative jelly strengths obtained were: unboiled, 8·1 units; boiled, 5·5 units. The loss of jellying power was less than is sometimes found; however, it was sufficient to show that the value of the dissociation "constant" of the acid groups is not materially affected by changes in the jellying power of the pectin.

The effect of partial de-esterification was studied in the case of a third orange pectin. Part of a solution of the pectin was treated with an amount of NaOH sufficient only for partial saponification. The pectin was then separated in the ash-free condition in the usual manner, and at the same time the remainder of the original pectin was separated similarly. The partial neutralization data are

given in Table 4. (The metallic constituents had not been removed so thoroughly as usual from these pectins, and allowance has been made for this as already described.)

Table 4. *Effect of partial de-esterification on the neutralization curve and dissociation of a pectin*

Orange pectin No. 3

		Original	Partly de-esterified
Analytical details of pectin	Free acidity	4·23	7·50 (as NaOH % of calcium pectate yield)
	Saponification value	13·91	10·35 (as NaOH % of calcium pectate yield)
	Alkalinity of ash	0·16	0·17 (as NaOH % of calcium pectate yield)
	Equivalent weight	911	522

	Concentration of pectin				
No.	g. per 100 g. water	g. equiv. per 1000 g. water	Degree of neutralization	*p*H	$K \times 10^{-4}$
Original:					
1	1·06	0·0116	0·036	2·67	6·0
2	1·06	0·0116	0·176	2·92	4·7
3	1·06	0·0116	0·396	3·35	3·6
4	1·06	0·0116	0·589	3·85	2·1
Partly de-esterified:					
5	1·06	0·0204	0·022	2·53	5·9
6	1·06	0·0204	0·245	3·00	4·2
7	1·06	0·0204	0·381	3·29	3·5
8	1·06	0·0204	0·512	3·60	2·8

The values of K, when plotted against degree of neutralization, lie on the same curve for the two pectins, in spite of the appreciable difference in the equivalent concentrations. This confirms the tendency noted earlier, in connexion with the red currant pectin of Table 1, for K to approach a constant value (for a given degree of neutralization) at higher concentrations.

The comparative uniformity of the dissociation properties of the various specimens and grades of pectin would appear to justify the view that the dissociation constant is a characteristic of the galacturonic acid unit in the molecule and not of any particular proportion or arrangement of esterified and unesterified units.

The dissociation of a pectin in presence of a second acid was examined by comparing the neutralization of a mixture of the two to a given *p*H with that of the second acid alone to the same *p*H. The following calculation applies to these conditions.

Let $[T]_P$ and $[T]_A$ be the total equivalent concentrations of the pectin and of the second acid;

$[Na]_1$ the equivalent concentration of alkali required to bring the H-ion concentration of the mixture to a given value $[H^+]$;

[HP] and [HA] the equivalent concentrations of undissociated pectin and of undissociated second acid at that value of $[H^+]$; and

$[Na]_2$ the equivalent concentration of alkali required to bring the second acid only (in the same concentration as in the mixture but in a separate solution) to the same H-ion concentration.

Then in the mixture, assuming complete dissociation of the alkali salts, with sufficient exactness

$$[T]_P + [T]_A = [HP] + [HA] + [Na]_1 + [H^+]. \qquad \text{.....(1)}$$

In the solution of the second acid only

$$[T]_A = [HA] + [Na]_2 + [H^+]. \quad \ldots\ldots(2)$$

The values of [HA] in (1) and (2) are the same if it be accepted that the dissociation constant of the second acid is unaffected by the pectin, and hence, subtracting (2) from (1),

$$[T]_P = [HP] + [Na]_1 - [Na]_2. \quad \ldots\ldots(3)$$

For the pectin, at the *p*H in question and for the concentration used,

$$K = \frac{[H^+]\,([T]_P - [HP])}{[HP]}.$$

Hence from (3),

$$K = \frac{[H^+]\,([Na]_1 - [Na]_2)}{[T]_P - ([Na]_1 - [Na]_2)}. \quad \ldots\ldots(4)$$

Table 5 shows the neutralization data obtained with mixtures of a red currant pectin and citric acid, and the parallel data for the citric acid only which provide the values of $[Na]_2$.

Table 5. *Dissociation of pectin in presence of citric acid*

Red currant pectin No. 3

Analytical details of pectin: Free acidity 5·33% (as NaOH % of calcium pectate yield); Saponification value 12·44% (as NaOH % of calcium pectate yield); Alkalinity of ash 0·00% (as NaOH % of calcium pectate yield); Equivalent weight 750

Neutralization of citric acid only

g. equiv. per 1000 g. water		
Citric acid $\times 10^{-4}$	Alkali $\times 10^{-4}$	*p*H
67·0	0	3·00
67·0	10·2	3·33
67·0	20·5	3·85
134·0	0	2·80
134·0	20·5	3·20
134·0	41·0	3·78

Neutralization of mixtures of pectin and citric acid

g. equiv. per 1000 g. water						
Pectin $\times 10^{-4}$	Citric acid $\times 10^{-4}$	Alkali $\times 10^{-4}$	*p*H	Equiv. conc. of alkali for same *p*H with citric acid only (by interpolation) $\times 10^{-4}$	$[H^+]$ $\times 10^{-4}$	K (by equation (4)) $\times 10^{-4}$
67·0	67·0	20·5	3·04	1·2	9·12	3·7
67·0	67·0	41·0	3·40	11·6	3·98	3·1
67·0	134·0	30·8	3·02	11·4	9·55	3·9
67·0	134·0	61·5	3·45	30·7	3·55	3·0

The values of K for the pectin in these mixtures are in reasonably good agreement with those for red currant pectin alone at the same concentration and *p*H (cf. Table 1). It appears therefore that the dissociation of the pectin is not materially altered in dilute solutions by the presence of another weak acid and its salt, assuming that the dissociation of this other acid is not altered by the pectin.

This is an important result, for it governs the case of pectin in admixture with other acids and salts such as are present in fruit juices and which therefore usually accompany the pectin in the process of jellying. Advantage has been taken of this in the next section in dealing with buffered jellying mixtures.

The relation between the jellying of pectin and its dissociation

Experiments were undertaken with the object of studying the jellying of pectin in the light of the data established for its dissociation.

It is familiar that in jellying mixtures containing pectin there is, under given conditions of *p*H and total solids concentration, a minimum concentration of pectin which must be present for jellying to occur [Hinton, 1939, p. 16]. Mixtures with such minimum concentrations of pectin seemed to offer a desirable basis for these experiments, having a twofold advantage. On the one hand they can be regarded as jellies with a zero jelly strength, and on the other hand they are still solutions of pectin and so are subject to the dissociation behaviour already established.

For convenience of experiment these mixtures were prepared by fixing the composition as regards the concentrations of pectin and total solids, and adjusting the *p*H by trial in a series of mixtures by means of buffer solutions, so as to cover a range which would include the *p*H at which jellying just failed. Details were as follows.

Buffer solutions were prepared having *p*H values at intervals of 0·04 by partial neutralization of a lactic acid solution with KOH. (Lactic acid was used in preference to citric or malic acids because of the greater solubility of the Ca salt, required in experiments to be reported later.) A quantity of a mixed syrup of sucrose and invert sugar at 100–105° was mixed with a suitable amount of pectin paste momentarily brought to a temperature of about 95°, and the mixture was quickly poured into a series of boiling-tubes containing the required amounts of a sequence of the buffer solutions below and above the expected highest *p*H for setting. The pectin-sugar mixture was added up to the level of a mark on the tubes, and the latter were at once stoppered and the contents mixed. The tubes were placed in a refrigerator at about 5° and kept there for a week. Then, by inspection, those mixtures immediately above and below the jellying point were picked out, their *p*H determined and an estimate made of the probable exact value of the highest *p*H for jellying. (The *p*H values were determined on solutions of the mixture diluted with an equal weight of water, for greater sensitivity in the readings. By comparison with several of the less viscous undiluted mixtures, it was found that a deduction of 0·08 from the *p*H of the 50 % solution gave the value of the undiluted mixture.) The concentration of total solids was checked by a refractometer reading.

The amounts of the buffer solutions used were such that the total (free and combined) lactic acid in the mixtures was 0·6 %. The invert sugar amounted to about 30 % of the mixture. The results for two series of mixtures containing de-ashed red currant and raspberry pectins are given in Table 6.

Although these results are presented in the form of the upper limit of *p*H for jellying at certain concentrations of total solids and of pectin, they may equally well be regarded as giving the minimum pectin concentration for jellying at the respective *p*H values and total solids concentrations. From this viewpoint the mixtures represent a solubility or saturation limit beyond which insoluble pectin substance would separate as jelly. As they stand, these limits of solubility clearly vary considerably with *p*H for a given concentration of total solids. They must, however, be taken as being *total* solubilities at the respective *p*H values; that is, they include those pectin molecules containing dissociated acid groups (i.e. ionized pectin), as well as those molecules containing no dissociated groups. But according to the ideas developed in the

Table 6. *Upper limit of* p*H for jellying.* (*Mixtures buffered with potassium lactate-lactic acid*)

Analytical details of pectins:

	As NaOH % of calcium pectate yield			
	Free acidity	Saponification value	Alkalinity of ash	Equivalent weight
Red currant No. 4	4·47	13·38	0·28	842
Raspberry	4·28	13·23	0·34	865

	Total solids concentration		Pectin concentration			
No.	% by weight	g. per 100 g. water	% by weight	g. per 100 g. water	g. equiv. per 1000 g. water	*p*H at which jellying just failed
Red currant:						
1	65·8	192	0·40	1·17	0·0139	3·62
2	65·8	192	0·60	1·76	0·0208	3·75
3	69·7	230	0·20	0·66	0·0078	3·57
4	69·7	230	0·30	0·99	0·0118	3·71
5	69·7	230	0·40	1·32	0·0157	3·80
6	69·7	230	0·50	1·65	0·0196	3·86
7	72·8	268	0·12	0·44	0·0052	3·76
Raspberry:						
1	64·0	178	0·50	1·39	0·0160	3·57
2	67·0	203	0·35	1·06	0·0123	3·62
3	67·0	203	0·50	1·51	0·0175	3·74
4	70·0	233	0·20	0·67	0·0077	3·64
5	70·0	233	0·35	1·17	0·0135	3·73
6	70·0	233	0·50	1·67	0·0193	3·82
7	72·6	265	0·20	0·73	0·0084	3·79
8	72·6	265	0·35	1·28	0·0148	3·88

introduction it is only the latter which are capable of entering into jelly formation, and hence it is the *partial* solubility (in the sense used by Michaelis [1926]) of this non-ionized pectin which must be regarded as reaching a solubility limit in each of these mixtures.

At this point it is necessary to take account of a fundamental peculiarity of the pectin molecule, viz. the possession of a variable degree of acidity or equivalent weight. This arises, of course, from the varying degree of esterification it may have. For the "effective" ionization of a molecule of given chain

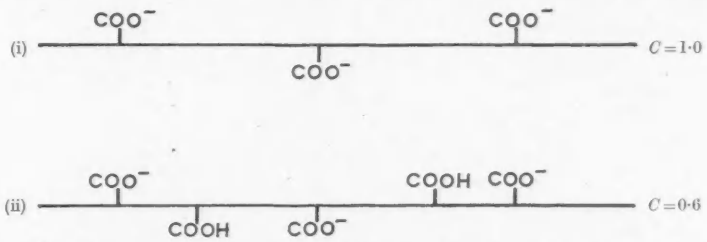

Fig. 2. Diagrammatic representation of critical degree of dissociation of the acid groups in the molecular chain of non-jellying molecules. (Esterified carboxylic groups not indicated.)

length to prevent it from participating in jelly formation, it would seem *prima facie* necessary and sufficient that there should be a particular spacing of ionized groups (dissociated acid groups) along the molecular chain (Fig. 2 (i)). Clearly

this spacing can only coincide with the spacing of the acid groups for one particular degree of esterification of the molecule (as at (i)).

At any lower degree of esterification, the acid groups in the chain will be more numerous and hence more closely spaced, and it will be unnecessary for every group to be dissociated to inhibit jellying. This condition is seen diagrammatically in Fig. 2 (ii); the three ionized groups assumed to be necessary for inhibition of jellying are still shown, but there are now two other groups exposed by de-esterification, and these need not necessarily be dissociated. In a molecule which is effectively ionized and therefore incapable of jellying, the ratio of dissociated to total (non-esterified) acid groups represents a critical minimum degree of dissociation within that molecule for its particular spacing of acid groups. This critical degree of dissociation in the ionized molecule will be called C; it is the ratio of dissociated to total unesterified acid groups in the effectively ionized molecule.

An alternative interpretation is to assume that all the acid groups of the molecule are dissociated, but that association occurs to a certain degree between these ionic groups and the K ions; C is then a factor which takes account of the extent of this association (which would be expected to vary with the spacing of the groups in the chain).

C may have values from 1 downwards, according to the degree of esterification of the molecule. In the mixture of variously esterified molecules constituting an ordinary pectin, C is unlikely to reach the value 1 for all molecules. The only way in which this critical proportion can be dealt with in a practical sense is as a statistical average of its separate values for the individual molecules, in the way in which other properties have been dealt with. In general, therefore, the average value of C for a pectin may be expected to be less than 1.

The data of Table 6 can now be examined with a view to evaluating C for particular cases.

Let $[P]$ be the concentration of total pectin in one of the mixtures (in g. equivalents per 1000 g. water). Suppose in the first instance that in the effectively ionized molecules (i.e. those incapable of jellying) all the acid groups are dissociated ($C=1$), and the equivalent concentration of this ionized pectin is $[P^-]$. Then we have

$$K=\frac{[H^+]\,[P^-]}{[P]-[P^-]},$$

whence

$$[P^-]=\frac{K\,[P]}{K+[H^+]}.$$

But according to the hypothesis put forward, in general only a fraction C of the acid groups in the effectively ionized pectin molecules is dissociated. Hence the actual concentration of the latter is

$$\frac{[P^-]}{C}=\frac{K\,[P]}{C\,(K+[H^+])}. \qquad \text{.....(5)}$$

This may now be converted for convenience into the form g. per 100 g. water. If p is the concentration of total pectin expressed in this form, then that of the effectively ionized pectin (expression (5)) becomes

$$\frac{K\,[P]}{C\,(K+[H^+])}\times\frac{p}{[P]}\text{ g. per 100 g. water,}$$

which simplifies to

$$\frac{Kp}{C\,(K+[H^+])}. \qquad \text{.....(6)}$$

We can return now to the consideration of the non-ionized pectin, i.e. that which is capable of jellying but which in these mixtures does not in fact do so

because it is present only at its solubility limit. Its concentration, s, is clearly the difference between the total and the ionized pectin, viz.

$$s = p - \frac{Kp}{C(K+[H^+])} \text{ g. per 100 g. water.} \qquad \text{.....(7)}$$

The values of K applicable can be read off with sufficient exactness from Fig. 3, which has been plotted from the data for the red currant pectin of Table 1 to facilitate selection of K values appropriate to the pH and pectin concentration

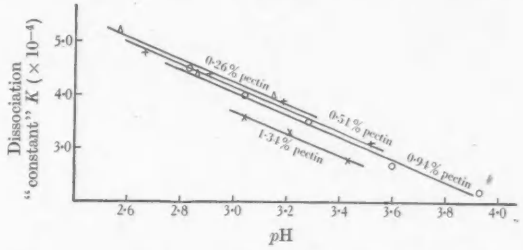

Fig. 3. Variation of dissociation "constant" with pH at several concentrations of pectin. (Red currant pectin No. 1.)

0·26 g. pectin per 100 g. water ×
0·51 g. pectin per 100 g. water o
0·94 g. pectin per 100 g. water +
1·34 g. pectin per 100 g. water △

of the mixtures. Taking the first two mixtures of Table 6, the K values are $3{\cdot}0 \times 10^{-4}$ and $2{\cdot}8 \times 10^{-4}$ respectively. Hence from (7), the concentrations or solubilities of non-ionized pectin are

$$1{\cdot}17 - \frac{0{\cdot}65}{C} \text{ and } 1{\cdot}76 - \frac{1{\cdot}08}{C}.$$

The total solids concentrations in these two mixtures were identical, as also were the temperatures, and it seems a not unreasonable assumption that the solubility of this non-ionized pectin is the same in both cases.

Equating the two expressions, therefore,

$$1{\cdot}17 - \frac{0{\cdot}65}{C} = 1{\cdot}76 - \frac{1{\cdot}08}{C}$$

whence $C = 0{\cdot}73$.

The same method of evaluating C may now, by taking them in pairs, be applied to the four red currant pectin mixtures Nos. 3–6 of Table 6, which each had the same concentration of total solids, and to the raspberry mixtures Nos. 2–8. The data and results of these calculations are shown in Table 7.

The differences between the several values of C obtained for each pectin are not significant in view of the limits of accuracy of the observations and quantities involved. Hence it is permissible to take the averages as being more correct than the individual results. These averages are shown in the table, and it appears that they are practically identical for the two pectins.

It is now possible to substitute the average value of C in the formula (7) for the concentration of non-ionized pectin. This has been done for each mixture,

and the results are shown in the last column of Table 7. These concentrations are the solubilities of the non-ionized pectin in mixtures with total solids concentrations as shown in Table 6, and at a temperature of approximately 5°.

Table 7. *Critical dissociation of pectins and solubilities of the non-ionized pectin*

No.	$[H^+] \times 10^{-4}$ (at upper limit for jellying)	K (from Fig. 7) $\times 10^{-4}$	Concentration of ionized pectin (from (6)) g. per 100 g. water	C (by equating values for (7))	Solubility of non-ionized pectin g. per 100 g. water
Red currant:					
1	2·40	3·0	$\frac{0·65}{C}$	0·73	0·28
2	1·78	2·8	$\frac{1·08}{C}$		0·28
3	2·69	2·9	$\frac{0·34}{C}$	0·70	0·19
4	1·95	2·7	$\frac{0·57}{C}$	0·76	0·21
5	1·58	2·6	$\frac{0·82}{C}$	0·73	0·20
6	1·38	2·5	$\frac{1·06}{C}$		0·20
7	1·74	2·3	$\frac{0·25}{C}$		0·10
			Average	0·73	
Raspberry:					
1	2·69	3·1	$\frac{0·74}{C}$		0·36
2	2·40	3·0	$\frac{0·59}{C}$	0·73	0·24
3	1·82	2·8	$\frac{0·92}{C}$		0·23
4	2·29	2·8	$\frac{0·37}{C}$	0·67	0·16
5	1·86	2·8	$\frac{0·70}{C}$	0·74	0·20
6	1·51	2·7	$\frac{1·07}{C}$		0·18
7	1·62	2·5	$\frac{0·44}{C}$	0·73	0·12
8	1·32	2·5	$\frac{0·84}{C}$		0·11
			Average	0·72	

It is evident that the partial solubility of the pectin decreases as the total solids concentration in the jellying mixture increases. The average values for the partial solubility at each total solids concentration are shown in Table 8, and are plotted against the total solids concentrations in Fig. 4.

The results for the two pectins fall substantially on the same curve.

Table 8. *Solubility of non-ionized pectin at varying total solids concentration*

Temperature approximately 5°

	Total solids g. per 100 g. water	Solubility of non-ionized pectin g. per 100 g. water
Red currant pectin	192	0·28
	230	0·20
	268	0·10
Raspberry pectin	178	0·36
	203	0·24
	233	0·18
	265	0·12

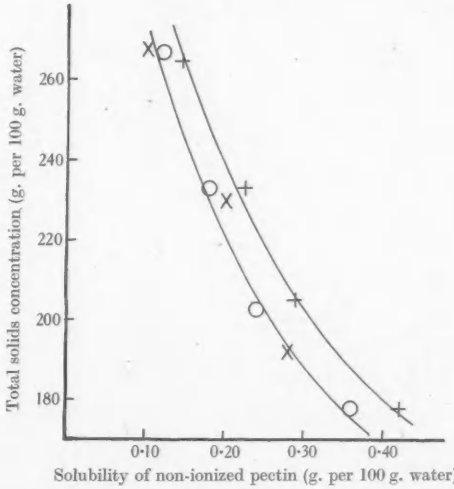

Fig. 4. Solubility of non-ionized pectin: variation with total solids concentration.

With potassium buffer { Red currant pectin 4 ×, Raspberry pectin o } With sodium buffers { Raspberry pectin + }

Comparison of effects of K and Na buffers

Jellies prepared with Na buffers are decidedly inferior in strength to similar jellies prepared with K buffers. In comparisons carried out for other purposes, jellies of a given rigidity could be obtained with K buffers at a *p*H about 0·2 higher than with Na buffers. It has already been seen that the dissociation "constant" of the pectin is the same when either of these metals is used as neutralizing agent.

Similar experiments to those already described were carried out to find the upper limit of *p*H for jellying in mixtures with Na lactate buffers. The raspberry pectin of Table 6 was used, and the data and results are shown in Table 9.

The value of C was found to be appreciably lower than in the K series. This means that a smaller proportion of the acid groups is dissociated for effective ionization of the molecule; in other words, the spacing of ionized groups in the molecular chain is wider in the Na series. This may be taken as evidence of a specific ionic association effect.

Table 9. *Critical dissociation of pectin and solubility of non-ionized pectin in raspberry pectin jellies with Na buffers*

No.	Total solids concentration: % by weight	Total solids concentration: g. per 100 g. water	Pectin concentration: % by weight	Pectin concentration: g. per 100 g. water	Pectin concentration: g. equiv. per 1000 g. water	*p*H at which jellying just failed	$[H^+] \times 10^{-4}$	K (from Fig. 7) $\times 10^{-4}$	Conc. of ionized pectin g. per 100 g. water (from (6))	Calculated value of C	Solubility of non-ionized pectin g. per 100 g. water
1	64·0	178	0·50	1·39	0·0160	3·37	4·27	3·5	$\frac{0·63}{C}$		0·42
2	67·0	203	0·35	1·06	0·0123	3·42	3·80	3·4	$\frac{0·50}{C}$	0·64	0·29
3	67·0	203	0·50	1·52	0·0175	3·52	3·02	3·3	$\frac{0·79}{C}$		0·29
4	70·0	233	0·35	1·17	0·0135	3·53	2·95	3·2	$\frac{0·61}{C}$	0·66	0·23
5	70·0	233	0·50	1·67	0·0193	3·62	2·40	3·1	$\frac{0·94}{C}$		0·22
6	72·6	265	0·20	0·73	0·0084	3·57	2·69	2·9	$\frac{0·38}{C}$	0·66	0·15
7	72·6	265	0·35	1·28	0·0148	3·68	2·09	2·9	$\frac{0·74}{C}$		0·14
									Average	$\overline{0·65}$	

The partial solubilities were rather higher than in the K series (v. Fig. 4), though the differences are possibly not greater than might be due to experimental error.

Alternative method for finding the solubility of the non-ionized pectin

A more direct method for finding the solubility of the non-ionized pectin consists in finding the limit of pectin concentration for jellying at a *p*H low enough for the dissociation of the pectin to be substantially suppressed.

Two mixtures of low pectin concentration were prepared from the raspberry pectin of Table 6, having in place of a buffer solution a mixture of lactic acid (the same proportion as in the earlier mixtures) and HCl to give a *p*H near 2. As this *p*H is approached $[H^+]$ becomes large in proportion to K, and the concentration of non-ionized pectin approaches the total pectin concentration (cf. formula (7)).

The procedure in preparing the mixtures was as before, and the data for their composition and condition as regards jellying, after a week in the refrigerator at approx. 5°, are shown in Table 10.

Table 10. *Jellying behaviour at low* p*H*

No.	Total solids concentration: % by weight	Total solids concentration: g. per 100 g. water	Pectin concentration: % by weight	Pectin concentration: g. per 100 g. water	Pectin concentration: g. equiv. per 1000 g. water	*p*H (50% solution)	Appearance
1	70·1	234	0·08	0·27	0·0034	2·25	Very weak jelly
2	70·6	240	0·05	0·17	0·0021	2·25	Syrup with only traces of jellying

It is evident that the pectin concentration of No. 2 was very close to the limiting value for jellying. Hence, in view of the suppression of ionization, the solubility of the non-ionized pectin can be but very little less than 0·17 g. per 100 g. water, even if a wide latitude for the values of K and C is allowed in applying formula (7). From Fig. 4 the partial solubility for a total solids concentration of 240 should be approximately 0·16, and with this figure the indications of the present experiment are in substantial agreement.

This method of finding the partial solubility of a pectin offers a means of avoiding certain complications such as the effect of the particular composition of the juice or buffer mixture on the ionization behaviour. This may make it unnecessary to separate the pectin in the ash-free condition in order to investigate its fundamental jellying properties.

Effect of partial de-esterification

Since de-esterification results in an increase in the proportion of acid groups in the molecule, and hence in the spacing of these groups in the molecular chain, it may be expected to give rise to changes in the critical degree of dissociation within the molecule, and hence in the jellying behaviour.

It has been shown above (Table 4) that de-esterification appears not to cause any appreciable change in the dissociation "constant" for a given degree of neutralization or pH. Evidence of a change in C was however found in the case of a red currant pectin prepared from crushed fruit, in which enzymic de-esterification had occurred. The equivalent weight of this pectin was 480. The upper limit of pH for jellying was determined as before with K lactate buffers. The amount of pectin available did not permit of determinations on more than a few mixtures; the results of these, with the calculated value of C and of the partial solubilities, are shown in Table 11.

Table 11. *Upper limit of* p*H for jellying of partly de-esterified pectin*

Red currant pectin No. 5

Analytical details of pectin: Free acidity 7·82% (as NaOH % of calcium pectate yield); Saponification value 10·69% (as NaOH % of calcium pectate yield); Alkalinity of ash 0·51% (as NaOH % of calcium pectate yield); Equivalent weight 480

No.	Total solids concentration: % by weight	Total solids concentration: g. per 100 g. water	Pectin concentration: % by weight	Pectin concentration: g. per 100 g. water	Pectin concentration: g. equiv. per 100 g. water	pH at which jellying just failed	$[H^+] \times 10^{-4}$	K (from Fig. 7) $\times 10^{-4}$	Conc. of ionized pectin (from (6)) g. per 100 g. water	Calculated value of C	Solubility of non-ionized pectin g. per 100 g. water
1	66·0	194	0·20	0·59	0·0123	3·20	6·31	3·7	$\frac{0·22}{C}$	0·54	0·18
2	66·0	194	0·40	1·18	0·0246	3·38	4·17	3·5	$\frac{0·54}{C}$		0·18
3	70·0	233	0·20	0·67	0·0140	3·51	3·09	3·0	$\frac{0·33}{C}$		0·06

The value found for C was appreciably lower than with the pectins of higher equivalent weight (cf. Table 7), though the reduction was not in proportion to the lowering of the equivalent weight. This may perhaps be taken to show a greater tendency to jellying in the molecules of the de-esterified pectin, since a

narrower spacing of dissociated acid groups in the molecular chain is required to prevent it.

The solubility of the non-ionized pectin was also lower than in the more fully esterified pectins (cf. Table 8).

The jellying process in its quantitative aspects

In the case of a jelly of pure pectin and sugar, with no buffer present but with the pectin partly neutralized by alkali, the following constituents are involved in the electrolytic equilibria in the solution before jellying commences.

(i) Non-ionized pectin molecules (containing only undissociated acid groups).

(ii) Ionized molecules having a critical proportion C of their acid groups dissociated.

(iii) H ions.

(iv) Alkali metal ions.

(v) OH ions (in negligibly small concentration).

If the concentration of (i) is greater than the partial solubility at the particular total solids concentration and temperature of the mixture, the non-ionized pectin in excess will begin to separate and form jelly. Thereby the mass equilibrium will be disturbed, and adjustment must take place by union of H ions with anionic groups of the pectin (ii). This reinforces the concentration of non-ionized pectin (making more of it available for jellying), reduces the amount of ionized pectin and results in a rise of pH.

The separation of the excess of non-ionized pectin, and the accompanying reduction in the amount of ionized pectin and rise of pH, must continue until the concentration of the former has fallen to the level of its solubility. At this point, mass equilibrium still requires a definite relation between the concentrations of ionized and non-ionized pectin and of H ion; hence the solubility of the non-ionized pectin, in conjunction with the requirement for electrical neutrality, controls the final amount of pectin remaining in solution (i.e. unjellied) and also the final pH.

Thus, if s is the solubility of the non-ionized pectin, and p the total unjellied pectin (both as g. per 100 g. water), then from (7) above:

$$p = \frac{s}{1 - \frac{K}{C\,(K + [H^+])}}. \qquad \text{.....(8)}$$

Also the condition of electrical neutrality requires:

$$[H^+] + [Na^+] = [P^-] = \frac{K\,[P]}{K + [H^+]}, \qquad \text{.....(9)}$$

where [P] is now the concentration of unjellied pectin in g. equivs. per 1000 g. water (i.e. $[P] \times \frac{\text{equiv. wt.}}{10} = p$). From these equations both p and $[H^+]$ can be evaluated.

The rise of pH in unbuffered pectin jellies during the process of jellying can be demonstrated if the pH is determined, not on a solution of the jelly, but on the jelly itself.

In a jelly containing a buffer mixture, the case is similar except that, provided that the amount of buffer is large compared with the pectin, the pH cannot rise appreciably. Hence the value of $[H^+]$ is practically the same as the initial value (i.e. before jellying began); and since it is thus known with sufficient exactness, the evaluation of p is simplified, formula (8) alone being necessary.

In both the unbuffered and the strongly buffered jellies the non-ionized part of the finally unjellied pectin is the same in amount, being equal to the partial solubility of the pectin. The amount of the ionized part however is different in the two cases, since it is in equilibrium with different H ion concentrations. In the buffered jelly it is lower than in the unbuffered (other conditions being the same initially); consequently the excess of pectin which separates as jelly is greater, and the buffered jelly is stronger. This offers an explanation of a fact observed before the present work was begun, viz. that jellies prepared from purified pectin and depectinized fruit juice or a buffer solution were considerably stronger than jellies prepared from the purified pectin only, at the same initial *p*H.

Considering now the amount of pectin which separates as jelly, this is given by

$$= p_t - \frac{p_t - p}{1 - \frac{s}{C\,(K + [\mathrm{H}^+])}}, \qquad \text{.....(10)}$$

where p_t is the concentration (as g. per 100 g. water) of total pectin in the jellying mixture.

Values for p_t, s, C and $[\mathrm{H}^+]$ can be inserted in this formula from the known data. The value of K, however, should be that corresponding to the concentration not of the total pectin, but of the unjellied part, p, which is not known until K itself has been fixed. The values of K differ so little with concentration, however (v. Fig. 3), that it is possible to choose an approximate value of K, evaluate an approximate figure for p, and so arrive at a more precise value of K for use in formula (10).

For the purpose of examining the quantitative aspects of the strength of jellies on the basis established above, data for a number of jellies prepared from the pectins of Table 6 may be considered. These jellies were prepared with K lactate or Na lactate buffers having *p*H values below the upper limit for setting. The general method of preparation was similar to that used in preparing the earlier mixtures, except that the sugar syrup was added to a mixture of the buffer solution and pectin paste, and the whole poured into jelly-testing boxes. The jellies were tested after remaining overnight in a refrigerator (approx. 5°), the apparatus described by Campbell [1938] being used.

The compositions and strengths of the jellies are shown in Table 12, and in addition the amount of unjellied pectin as calculated by formula (8) and of jellied pectin by formula (10). The values of K shown are the more precise values arrived at after making a first approximation, as already described.

The pectin concentrations in these jellies are expressed on the basis of a constant weight of water (100 g.). Since it is now sought to compare pectin concentrations with jelly strengths, the latter should also be expressed on this basis. The jelly strength, however, expressed in terms of rigidity (g. weight per sq. cm.), has reference to unit area of jelly [Campbell, 1938], and the two modes of expression have to be reconciled in the following manner.

Suppose two jellies have the total solids concentrations x and y g. per 100 g. water. Then the weights of each containing 100 g. water are $100+x$ and $100+y$ g., and their volumes are $\frac{100+x}{d_1}$, and $\frac{100+y}{d_2}$ ml., d_1 and d_2 being the respective densities. The rigidities, were they to be compared on the basis of a constant weight of water, would therefore have to be expressed on areas equivalent to these volumes, or $\left(\frac{100+x}{d_1}\right)^{\frac{2}{3}}$ sq. cm. and $\left(\frac{100+y}{d_2}\right)^{\frac{2}{3}}$ sq. cm. re-

spectively. The rigidity of the second jelly would thus have to be expressed on an area $\left[\frac{(100+y)\,d_1}{(100+x)\,d_2}\right]^{\frac{2}{3}}$ times that on which the rigidity of the first was expressed. Hence, if the rigidity of the first is determined on the usual basis as g. per sq. cm., that of the second, as determined on the same basis, would have to be multiplied by $\left[\frac{(100+y)\,d_1}{(100+x)\,d_2}\right]^{\frac{2}{3}}$ for the purpose of comparison with the pectin concentration.

For present purposes, the densities of the jellies may be taken as equal, $d_1=d_2$. Also, if x is 233, as in Nos. 3–8 of Table 12, the multiplying factor for other jellies becomes

$$\left[\frac{100+y}{333}\right]^{\frac{2}{3}}.$$

For $y=192$, the factor is 0·92 and for $y=268$, it is 1·07.

These corrections have been applied to the rigidities of Table 12 where necessary.

Table 12. *Comparison of amount of jellying portion of pectin with strength of jelly obtained*

No.	g. per 100 g. water: Total solids	g. per 100 g. water: Pectin p_t	*p*H of jelly	[H] $\times 10^{-4}$	K $\times 10^{-4}$	g. per 100 g. water: s (from Tables 8 and 9)	g. per 100 g. water: Unjellied pectin (from (8)) (p)	g. per 100 g. water: Jellied pectin (from (10))	Jelly strength (corrected) (units of rigidity)	Ratio: Jelly strength / Jellied pectin
					Red currant (K buffers)					
1	192	1·18	3·14	7·24	3·8	0·28	0·53	0·65	15·6	24
2	192	1·18	3·30	5·01	3·5	0·28	0·64	0·54	11·5	21
3	233	0·67	3·10	7·94	3·7	0·19	0·34	0·33	7·0	21
4	233	0·67	3·25	5·62	3·4	0·19	0·40	0·27	6·0	22
5	233	0·67	3·40	3·98	3·2	0·19	0·49	0·18	3·3	18
6	233	1·33	3·19	6·46	3·5	0·19	0·37	0·96	20·5	21
7	233	1·33	3·39	4·07	3·1	0·19	0·47	0·86	19·2	22
8	233	1·33	3·54	2·88	2·9	0·19	0·61	0·72	13·0	18
9	268	0·73	3·22	6·03	3·0	0·10	0·18	0·55	12·3	22
10	268	0·73	3·40	3·98	2·7	0·10	0·22	0·51	11·1	22
11	268	0·73	3·50	3·16	2·6	0·10	0·26	0·47	8·8	19
					Raspberry (K buffers)					
1	233	0·67	3·05	8·91	3·7	0·18	0·30	0·37	4·2	11
2	233	0·67	3·29	5·13	3·3	0·18	0·39	0·28	2·0	7
3	233	1·33	3·10	7·94	3·6	0·18	0·32	1·01	13·8	14
4	233	1·33	3·36	4·36	3·2	0·18	0·44	0·89	10·2	11
					Raspberry (Na buffers)					
1	233	1·33	3·28	5·25	3·5	0·22	0·57	0·76	3·3	4
2	233	1·33	3·10	7·94	3·7	0·22	0·43	0·90	5·8	6

The results appear to give some indication of a direct quantitative basis for the jelly strength. In the final column of the table the ratio of the jelly strength to the concentration of jellied pectin is shown; for each pectin and type of buffer salt there is some approach to a constant ratio. Although the jelly strengths would presumably have increased somewhat on prolonged storage, probably the approach to constancy of this ratio would not be materially affected. Indeed, there is some possibility that it would be improved, as a tendency will be noted for the recorded ratio to be low where the *p*H is rather high. Morris [1935] has shown that the rate of setting of pectin jellies may be delayed by raising the *p*H by addition of a buffer salt. In the present series, the concentration of buffer

salt is obviously greater where the *p*H is higher, and hence a greater relative rise in jelly strength, and therefore in the ratio, might be expected on longer standing in those cases where the latter is at present low.

Accepting the constancy of this ratio, therefore, it may be taken to mean that when allowance has been made for the solubility of the non-ionized pectin and for the ionized pectin in equilibrium with it, the rest of the pectin builds up a jelly structure whose strength is directly proportional to the amount of pectin composing it.

The ratio for the raspberry pectin (with K buffers) is only about half that for the red currant pectin. This would appear to indicate an intrinsic difference between the jellying powers, not merely of the entire pectins, but of the portions actively engaged in jelly formation. Such an intrinsic difference is clearly independent of the arbitrary conditions of *p*H, total solids concentration and pectin concentration, and is likely to be related to some fundamental property such as the degree of polymerization of the molecule.

Some consequences p*H-jelly strength relations*

As an outcome of the present work four characteristics of pectins have been found which are effective in regulating the strengths of jellies under particular conditions:

(1) Dissociation of the acid groups in accordance with an apparently variable dissociation "constant".

(2) A critical degree of dissociation of the acid groups of the individual molecule for effective inhibition of the jellying propensity.

(3) The solubility of the non-ionized pectin.

(4) The intrinsic jellying capacity of the non-ionized pectin present in excess of the solubility limit.

An examination of the possible variations and combinations of these factors shows that they can account to a considerable degree for the main features of the jellying behaviour of pectins under various conditions.

The general relation between *p*H and jelly strength, for example, when premature setting is avoided, is expressed by a maximum jelly strength in the neighbourhood of *p*H 2 or somewhat below, with the strength declining as the *p*H is raised, eventually more or less sharply as the upper limit of *p*H for jellying is approached [Tarr, 1926; Lüers & Lochmüller, 1927; Olsen, 1934; Lampitt & Money, 1939]. This fall towards the upper limit tends to be sharp when the pectin concentration is high, and more gradual when it is lower [Stuewer *et al.* 1934]. The difference is one which can be seen to be necessitated by the interplay of the four characteristics of the pectin. Thus, a case may be taken where

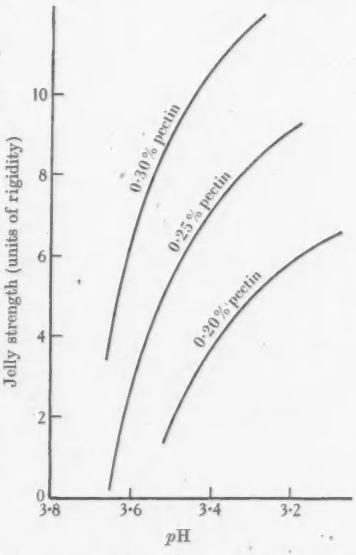

Fig. 5. Typical calculated *p*H-jelly strength curves at varying pectin concentration.

$C=0{\cdot}73$, the partial solubility is 0·20, and the jelly strength ratio is 21, a combination of figures such as was found for the red currant pectin of Tables 6 and 12. Jelly strengths corresponding to these data can be calculated for any total pectin concentration p_t and at any pH by means of formula (10) above, the values of K appropriate to the pH being taken from Fig. 3. Fig. 5 shows the pH-jelly strength curves calculated in this way for three concentrations of pectin; it can be seen that the slope towards the upper limit of pH is much steeper for 0·30 % than for 0·20 % pectin. The general resemblance of these curves to those of Stuewer *et al.* [1934, Fig. 5] is clear, considering only those portions of their curves which are above the "optimum" pH. In a separate communication by Olsen [1934], the occurrence of an optimum pH was shown to be an effect due to premature gelation. Had this been avoided, it is possible that the pH-jelly strength curves of Stuewer *et al.* would have been throughout in general conformity with the requirements of the present hypothesis. (Detailed conformity cannot of course be checked in the absence of knowledge as to the particular numerical magnitudes involved.)

The fact that, when premature setting is avoided, the jelly strength exhibited at the pH of 2 is not far from the maximum attainable is another relation necessitated by the factors under discussion. It is this relation which makes it desirable to compare the jellying powers of pectins at this pH [Hinton 1939, p. 16]. Taking formula (10) above for the amount of pectin capable of separating as jelly, the limit when $[H^+]$ is very large compared with K is

$$p_t - s. \qquad \text{.....(11)}$$

At a pH of 2, taking round figures of 5×10^{-4} for K and 0·7 for C, the amount capable of jellying is

$$p_t - 1{\cdot}07\,s. \qquad \text{.....(12)}$$

For jellies containing usual concentrations of pectin, the difference between (11) and (12) is hardly greater, proportionally, than the possible experimental error of the jelly strength determination. Thus, in the comparisons of jellying power discussed elsewhere [Hinton, 1939, Parts IV and V], since the concentration of pectin was 0·35 % and the water content 35 %, $p_t=1{\cdot}00$; and if s is assumed to be as high as 0·30, expressions (11) and (12) take the values 0·70 and 0·68 respectively. Hence the jellying powers recorded at pH 2 should be within about 3 % of the theoretical maximum. The discrepancy of course would be more significant if the pectin concentration were low, or if the value of s were raised by increasing the water content.

Another direction in which jellying behaviour is interpreted is in regard to the effect of total solids concentration. The effect of lowering the latter is to shift the pH-jelly strength curve downwards to a lower pH range, the shift becoming more marked as the total solids concentration becomes lower [Stuewer *et al.* 1934]. Taking once more the case of the red currant pectin above, Fig. 6 shows the curves calculated for three concentrations of total solids, for jellies containing 0·25 % pectin. The resemblance to the relevant portions of the curves of Stuewer *et al.* [1934, Fig. 3] is again evident, and in this case also the conformity of the latter with the present ideas might have been extended to the whole curves had premature gelation been avoided.

The possibility of variable values for C and s provides for an explanation of a further phenomenon to which some attention has already been given [Hinton, 1939, p. 66]. In the case of two pectins which only differ in their degrees of esterification, the more highly esterified pectin forms jellies up to a higher limit of pH than the other, yet its jellies may at a low pH be slightly weaker; there is

a crossing over of the *p*H-jelly strength curves. The adequacy of the factors under discussion to account for an effect of this kind can be seen if the full *p*H-jelly strength curves are plotted for a particular case. For this purpose the

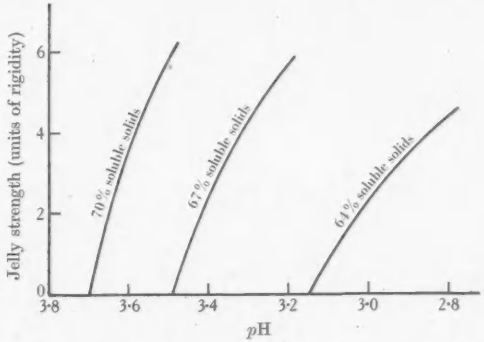

Fig. 6. Typical calculated *p*H-jelly strength curves at varying total solids concentration.

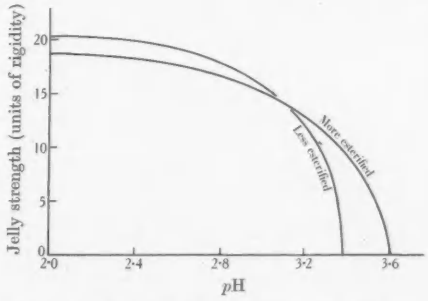

Fig. 7. Typical calculated *p*H-jelly strength curves at different degrees of esterification of the pectin.

data found for the red currant pectins of Tables 7 and 11 may be used. Fig. 7 shows the curves for these pectins at a concentration of 0·4 %, with 66 % of total solids. The rigidity factor of 21 (v. Table 12) has been used in calculating the jelly strengths from the amounts of jellied pectin as given by formula (10). The curves, it can be seen, reproduce the essential features of the diagrammatic curves previously suggested. The calculation brings out the reason for the crossing over, viz. the decrease in the solubility (*s*) of the non-ionized pectin which accompanies the fall in the value of *C* resulting from the de-esterification.

Attention has already been drawn to the fact that the upper limit of *p*H for setting is raised when the degree of esterification is increased [Hinton, 1939, p. 65]. This rise of the *p*H limit can be accounted for if the critical dissociation *C* is assumed to become greater with increasing esterification. This assumption is a justifiable one in view of the demonstration (cf. Tables 7, 11) that *C* becomes smaller with partial de-esterification, as well as on the *prima facie* grounds already discussed (p. 1220).

The magnitude of this rise in the value of C can be approximately evaluated in the case of a pectin which was prepared by esterifying an orange pectin with methyl alcohol. The equivalent weights of the pectin before and after the esterification were 615 and 1480 respectively. Jellies were obtainable up to a pH of about 4·2, whereas the limit for the untreated pectin under similar conditions was 3·4 (total solids, 70%; pectin, 0·21%, $p=0{\cdot}70$; K citrate buffers; temperature, approx. 10°). For the untreated pectin a value for C of 0·62 may be taken (i.e. linearly intermediate between the values for the pectins of Tables 7 and 11, with equivalents of 842 and 480). This, with $K=3{\cdot}3\times10^{-4}$, gives the solubility (s) of the non-ionized pectin under the conditions of the test, by formula (7), as 0·19 g. per 100 g. water. (This is a not unreasonable figure, compared with a value suitably intermediate between the earlier results of Tables 8 and 11, in view of the slightly higher temperature in this case.)

If no increase in s occurred as a result of the esterification, then C can be approximately calculated for the esterified pectin by applying formula (7). Taking K as approximately $1{\cdot}6\times10^{-4}$ (by extrapolation from the data of Table 4), the formula gives

$$0{\cdot}19=0{\cdot}70-\frac{1{\cdot}6\times10^{-4}\times0{\cdot}70}{C\,(1{\cdot}6+0{\cdot}6)\times10^{-4}},$$

whence

$$C=1{\cdot}0.$$

This means that for effective ionization of the pectin molecules when the equivalent weight reaches about 1480 all the acid groups must be dissociated.

Had s been increased by the esterification, the calculation would give C as greater than 1, which would be meaningless. It seems, therefore, that s is unlikely to have increased appreciably. On the other hand, it is unlikely to have decreased, in view of the fact that a decrease was found to accompany de-esterification.

It may be pointed out before concluding that it has been the intention in this paper to demonstrate the general principles governing the separation of pectin as jelly, rather than to claim any absolute numerical validity for the factors and quantities evaluated from the experimental work. The numerical values obtained are, in fact, probably affected to some extent by certain experimental approximations which have been made.

For example, in the calculations of the latter part of the paper the appropriate values for the dissociation "constant" have been taken from Fig. 3, which gave the values for one particular pectin. Although other pectins have been found to have substantially the same system of "constants" as this, yet minor variations cannot be ruled out altogether; and if the values for this pectin are not strictly applicable to others, the calculated values of the jellying factors will be affected. To take an illustration, if the values of K used in Table 7 are taken from the extrapolated data for the orange pectin of Table 1, the numerical value of C averages 0·82, and the results for the solubility of the non-ionized pectin are somewhat increased. These changes, however, do not affect the general argument as to the factors controlling the extent of jelly formation under given conditions.

Again, in finding the upper limit of pH for jellying, the pectin mixtures were allowed to stand for a week to develop any signs of jellying. Had they been left longer it is possible that slightly higher limits might have been found. The pH limit after one day only was observed in a number of the mixtures, and was never more than about 0·1 below the figure after a week; hence further changes of any magnitude would seem unlikely. Any further small upward shifts of the

pH limits would tend to give slightly higher values for C, and slightly lower solubilities for the non-ionized pectin. Again, however, the general nature of the results would be unaffected.

Summary

The electrolytic dissociation of pectins in dilute solution was investigated. The dissociation "constant" was found to diminish with increasing degree of neutralization with alkali, from a value of about 5×10^{-4} for the free acid condition in a concentration of approx. 1 g. per 100 g. water.

Dilution caused a small decrease in the value of K at a given pH or degree of neutralization.

No significant alteration of the dissociation "constant" was caused by boiling a pectin solution for 1 hr., or by enzymic de-esterification or by admixture with citric acid.

From the results of determinations of the upper limit of pH for the jellying of pectin-sugar mixtures on the assumption that jelly formation is only participitated in by molecules in the non-ionized condition, that it is shown that the concentration of such non-ionized pectin must exceed a certain solubility, or saturation limit, which varies with the total solids concentration of the mixture. It is necessary to assume that in general the pectin which is ionized and incapable of jellying has a certain proportion only of its acid groups dissociated. This proportion varies with the nature of the buffer salt present (potassium or sodium), and with the degree of esterification of the pectin. The strengths of jellies from any one pectin, with a given buffer salt, were found to be proportional to the amount of the excess of non-ionized pectin above the limit of its solubility. For different pectins, the ratios of jelly strength to amount of jellying substance differed.

It is shown that many of the phenomena of the jellying of pectins, especially in regard to pH conditions, can be accounted for by the proposed hypothesis.

This work was carried out in the laboratories of the British Association of Research for the Cocoa, Chocolate, Sugar Confectionery and Jam Trades, and the author wishes to express his thanks to the Director of Research, Mr Thos. Macara, and the Council of the Association for permission to publish this paper.

Acknowledgement should be made of the assistance of Mr E. H. Steiner in part of the experimental work.

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146. THE FATE OF OXALOACETATE IN ANIMAL TISSUES

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(*Received 1 July 1940*)

According to the theory of the citric acid cycle the following anaerobic reactions are expected to take place when oxaloacetate is added to pigeon breast muscle [see Krebs, 1940].

(1) Oxaloacetate + "triose" → pyruvate + malate.

(2) 2 Oxaloacetate + pyruvate → citrate + CO_2 + malate.

(3) Oxaloacetate + citrate → α-ketoglutarate + CO_2 + malate.

The net effect of these reactions is:

(4) 4 Oxaloacetate + triose → 3 malate + α-ketoglutarate + 2 CO_2.

In addition to 1, 2 and 3, occurs the non-enzymic decomposition of oxaloacetate:

(5) Oxaloacetate → pyruvate + CO_2,

and this reaction can provide the pyruvate required in reaction 2. If 1 is completely replaced by 5, the net effect of 5, 2 and 3 is

(6) 4 Oxaloacetate → 2 malate + α-ketoglutarate + 3 CO_2.

In these reactions, malate stands for the system fumarate $\rightleftharpoons$ *l*(−)malate, "citrate" for the system citrate $\rightleftharpoons$ *cis*-aconitate $\rightleftharpoons$ *iso*citrate, and "CO_2" for the system $CO_2 \rightleftharpoons HCO_3'$.

The experimental results obtained with pigeon breast muscle, pigeon liver, sheep's heart and guinea-pig kidney, agree well with scheme 6 and this is further evidence in favour of the occurrence of the citric acid cycle in these tissues.

In brain and testis (tissues in which the occurrence of the citric acid cycle has been uncertain) very similar results were obtained. Oxaloacetate forms in these tissues, as in muscle, α-ketoglutarate, as predicted by schemes 4 and 6. The obvious assumption is that the mechanism of the formation of ketoglutarate from oxaloacetate is the same as in muscle, i.e. that reactions 2 and 3 take place in these tissues, and it would then follow that the citric acid cycle also occurs in brain and testis. At the same time it is certain that other reactions take place in these tissues in addition to those formulated in the citric acid cycle and, as long as the nature and the extent of the other reactions are unknown, it seems premature to discuss the importance of the citric acid cycle in brain and in testis.

Methods

The methods used in this work were those described in previous publications [Krebs & Eggleston, 1940; Krebs, 1940; Krebs *et al.* 1940]. The tissue was suspended in phosphate saline and shaken anaerobically at 40° in the presence of oxaloacetate. After varying periods of incubation the amounts of succinate [Krebs, 1937], α-ketoglutarate [Krebs, 1938; 1940], fumarate and *l*(−)malate [Krebs *et al.* 1940], pyruvate [Westerkamp, 1933], citrate [Pucher *et al.* 1936] and CO_2 [Warburg & Yabusoe, 1924] were measured. Oxaloacetate was also

determined [Ostern, 1933; Edson, 1935] but no oxaloacetate was found in the tissue suspensions after incubation.

Anaerobic conditions were ensured by placing yellow phosphorus in the centre well of the cups. The oxaloacetate was measured into a side arm and added to the tissue suspension after a 10 min. period of shaking at 40°. During this period O_2 was completely removed from the system by the phosphorus.

A complete experiment required at least 10 conical manometer vessels provided with one or two side arms and a centre well; they were filled as follows:

Manometer no. ...	1, 2, 3	4, 5, 6	7, 8	9	10
Side arm 1	0·4 ml. 0·2 *M* oxaloacetate	—	—	0·2 ml. 0·2 *M* oxaloacetate	0·2 ml. 0·2 *M* oxaloacetate
Side arm 2	—	—	0·3 ml. 2 *N* HCl	0·3 ml. 2 *N* HCl	—
Centre well	Phosphorus	Phosphorus	Phosphorus	Phosphorus	—
Main compartment	4 ml. tissue suspension	4 ml. tissue suspension	4 ml. tissue suspension	2 ml. tissue suspension	4 ml. 0·5 *N* HCl
Gas	N_2	N_2	N_2	N_2	Air

The manometers were rapidly shaken at 40°. After 10 min., during which thermal equilibrium was established, the oxaloacetate was mixed with the contents of the main compartment in cups 1, 2, 3, 9 and 10, and the acid was added from the side arm in 7, but not in 8 and 9. No readings were taken in 1, 2, 3, 4, 5 and 6. These manometers were shaken for 1 hr., after which they were removed from the bath. The contents of 1 were rapidly poured into a 25 ml. measuring cylinder containing 2 ml. 10 % Na tungstate. The cup was rinsed with water (10–15 ml.) and the washings were added to the suspension. Enough H_2SO_4 was added to precipitate the protein (*p*H 5). The volume was made up to 25 ml. and filtered into another 25 ml. measuring cylinder. Succinic acid was determined in a measured volume of the filtrate. The contents of cup 2 were treated in the same way, but the filtrate was treated in the extractor with $KMnO_4$, in the presence of $MnSO_4$ and H_2SO_4, before succinic acid was extracted [see Krebs, 1940]. The result of the succinic determination in this cup represented the sum of the succinate and α-ketoglutarate present. Cup 3, in which fumarate and malate were to be determined, was emptied into a measuring cylinder kept at 40° and the water used for washing was also warmed to 40° in order to avoid a change in fumarate-malate equilibrium. To the combined suspension and washings 5 ml. 5 % metaphosphoric acid were added. Fumarate was then determined [Krebs *et al.* 1940]. Cups 4, 5 and 6 were controls to which no oxaloacetate was added; they were treated in the same way as 1, 2 and 3 respectively.

Readings were taken in 7, 8, 9 and 10, the first being made after 10 min. of thermal equilibration, before the addition of acid (in 7) or of the oxaloacetate (in 9, 10). A large rise in pressure occurred in 9, and it was therefore necessary to set the level of the meniscus in the left manometer limb at a very low level. Half-quantities of suspension and substrate were used in this cup, in order to keep the volume of gas produced within limits which could be measured.

Readings were continued at 10 min. intervals in 7, 8, 9 and 10. 5 min. before the end of the incubation period, the meniscus in 9 was reset at low level, to allow for the determination of the bound CO_2. The very small evolution of CO_2 during these 5 min. was calculated by extrapolation. At the end of the incubation period readings were taken again and the acid was added to the contents of 8 and 9. Readings in these two manometers were continued till the pressure

due to the liberation of the bound CO_2 became constant (10–15 min.). The contents of the cup were then made up to 25 ml. and used for the determination of pyruvate and citrate. 4 ml. of the 25 ml. were used for the determination of pyruvate by the carboxylase method. To adjust the reaction to *p*H 5·0, 0·095 ml. 3 *M* Na acetate (3 times equivalent to the HCl present) and 0·2 ml. 3 *M* acetate buffer *p*H 5·0 were added. The remaining solution was deproteinized with trichloroacetic acid and used for the determination of citric acid according to Pucher *et al.* [1936].

The CO_2 liberated in 7 was the bound CO_2 present in the suspension at the beginning of the experimental period. In 8 and 9 the production of free CO_2 during the experimental period, and the bound CO_2 present at the end were measured. In 10 the bound CO_2 present in the oxaloacetate solution was determined. In this cup a very rapid evolution of CO_2 due to bicarbonate was followed by slow rise in pressure due to the decarboxylation of oxaloacetic acid. The amount of bicarbonate was calculated by extrapolation. The calculated quantity and the bicarbonate found in 7 provided the data required for the calculation of the initial bound CO_2 in 9.

When further products were to be determined, e.g. lactate or oxaloacetate, more manometers, filled as 1 and 4, were used. The oxaloacetate solution was prepared immediately before use. The acid was neutralized with the calculated amount of *M* $NaHCO_3$ and freed from CO_2 in an evacuated Thunberg tube.

It is essential in these experiments that the metabolic reactions should be stopped rapidly at the end of the experimental period, and especially that the suspension should not come into contact with O_2 when the cups are detached from the manometer. To facilitate rapid handling, manometers 1–6 were placed in the bath at 2 min. intervals. This made it possible to deal with each manometer at once at the end of the incubation period.

Experiments on pigeon breast muscle

Formation of pyruvate from oxaloacetate. The formation of pyruvate from oxaloacetate, being a non-enzymic reaction, is independent of the metabolic activity of the tissue. If the tissue is "dead", owing to heating to 100° or to poisoning, pyruvate is formed quantitatively [see also Breusch, 1939]. If the tissue is metabolically active varying amounts of pyruvate appear depending on the type of tissue, on the proportion of tissue to oxaloacetate and on other experimental circumstances. The quantities of pyruvate formed are generally small, and sometimes even negligible when small "physiological" amounts of oxaloacetate are added. In our experiments it was necessary, however, to add comparatively large amounts in order to obtain effects which could be accurately measured. Therefore it was not possible to avoid completely the undesirable side reaction 5. Data showing the magnitude of the reaction are contained in Tables 2 and 4.

Since this paper is concerned with the *metabolic* reactions of oxaloacetate, a distinction is made between "oxaloacetate decomposed" according to 5 and "oxaloacetate metabolized". The amount of the latter is the difference between oxaloacetate added and pyruvate found. The yields of the metabolic products are calculated as percentage of the "oxaloacetate metabolized".

Formation of carbon dioxide. Large quantities of CO_2 are rapidly formed when oxaloacetate is added to muscle suspensions. In the example shown in Table 1, Q_{CO_2}, calculated for the first 20 min., equals 51. The bulk of the CO_2 cannot be formed by reaction 5 since no equivalent amounts of pyruvate appear. Of the total CO_2 of 1317 μl. only 436 μl. are accounted for by reaction 5 and

Table 1. *CO_2 formation from oxaloacetate in pigeon breast muscle*

300 mg. wet pigeon breast muscle in 4·0 ml. phosphate saline; oxaloacetate 0·02 *M*; "CO_2" includes bicarbonate and free CO_2. Method of Warburg & Yabusoe [1924].

Time after the addition of oxaloacetate min.	Total CO_2 formed in the presence of oxaloacetate (1792 μl.) μl.	Blank CO_2 μl.	Pyruvate formed from oxaloacetate μl.
5	320	6	—
10	588	8	—
20	1013	12	490
30	1224	13	—
40	1299	14	—
50	1321	14	—
60	1337	14	436

14 μl. by the blank. The amount of CO_2 due to metabolic reactions of oxaloacetate is thus 867 μl. or 65% of the oxaloacetate metabolized. Further data are given in Table 2.

Table 2. *Fate of oxaloacetate in pigeon breast muscle under anaerobic conditions*

The data refer to 4 ml. muscle suspension. Period of incubation 1 hr. The concentration of the suspension was in Exp. 1: 1 part muscle, 14 parts medium; in Exp. 2: 1 part muscle, 10 parts medium; in Exp. 3: 1 part muscle, 9 parts medium. Quantities in μl.

	Exp. 1		Exp. 2		Exp. 3	
	Control	Exp.	Control	Exp.	Control	Exp.
Observed:						
Oxaloacetate added	0	1792	0	1792	0	1344
Pyruvate found	0	436	0	260	0	582
CO_2	58	1327	129	1629	57	978
Succinate	46	56	0	220	52	109
α-Ketoglutarate	8	344	44	90	147	299
Fumarate + malate	37	1032	0	780	0	408
Citrate	6	22	0	32	—	—
Calculated:						
Oxaloacetate metabolized	1356		1532		762	
Yield of succinate	0·8%		14·3%		7·5%	
Yield of α-ketoglutarate	25%		5·9%		20·0%	
Yield of fumarate + malate	73%		51%		53·6%	
Yield of citrate	1·2%		2·1%		—	
Yield of CO_2	61·5%		81%		45·5%	
Recovery:						
Oxaloacetate metabolized, in μl. C	5440		6128		3048	
Sum of the products recovered, in μl. C	6449		6390		2948	
% recovery	120		104		97	

Balance sheet of oxaloacetate metabolism. Typical experiments in which the various metabolic products of oxaloacetate were determined, as described in the section "methods", are recorded in Table 2. The figure for CO_2 includes the free CO_2 formed during the actual experiment and the CO_2 liberated from bicarbonate after acidification, but the bicarbonate present in the oxaloacetate solution has been deducted. The figure for citrate includes *iso*citrate and *cis*-aconitate [see Krebs, 1940]. The lower part of the table contains the yields of the

metabolites, as percentage of the oxaloacetate metabolized. The figures were calculated as follows:

$$\text{yield} = \frac{\text{metabolite found with oxaloacetate—metabolite found in control}}{\text{oxaloacetate metabolized}} \times 100.$$

A special correction was necessary in the case of CO_2 to allow for the CO_2 formation by the non-metabolic reaction 5. The CO_2 formed in this way was taken to be equivalent to the pyruvate formed and the metabolic CO_2 was therefore calculated by deducting the pyruvate found from the total CO_2 found.

To calculate the total recovery of the oxaloacetate metabolized the quantities of all the substances were expressed in terms of "μl. carbon". The figures were obtained by multiplying "μl. substance" with the number of C-atoms in the molecule (Table 2).

The table shows the variations which occur in the type of experiment. The yield of α-ketoglutarate was usually near 25%. When a low yield was obtained, as in Exp. 2, a higher proportion of succinate and CO_2 was found. This suggests that reaction 6 was followed in this experiment by the conversion of α-ketoglutarate into succinate and CO_2 (see below). The yield of "malate" was usually near 50%, as expected according to 6; it was higher, up to 75%, in other experiments (e.g. Exp. 1). Such a yield is in accordance with 4. The yield of citrate is low (1–2%) because citrate formed reacts under the experimental conditions to form α-ketoglutarate.

The total recovery of the oxaloacetate metabolized was usually near 100%, within the limits of error. In Exp. 1 the recovery was 120% and this is to be expected if reaction 1 takes place and if scheme 4 holds. In this case 1 triose equivalent enters the reaction in addition to the 4 mol. of oxaloacetate and the total yield of carbon expressed in terms of oxaloacetate metabolized should be 119%.

The data thus agree, on the whole, with the schemes 4 and 6. It is probable that 4 holds when the tissue contains much of the reactive form of "triose", and 6 when "triose" is absent. Considerable variations in the concentrations of carbohydrates and their split products are very likely to be encountered in muscle suspensions.

Previous investigators [Breusch, 1939; Thomas, 1939] found roughly the same quantities of "malate", citrate and pyruvate. Their balance sheets were however incomplete since the formation of α-ketoglutarate, succinate and CO_2 was not measured.

Origin of succinate. In order to examine the origin of the succinate in the above experiment α-ketoglutarate and fumarate were added to muscle suspensions under the same conditions and succinate was determined after anaerobic incubation. Both substances form succinate [see also Weil-Malherbe, 1937] and these results do not therefore establish the source of succinate.

Table 3. *Formation of succinate from α-ketoglutarate and fumarate in pigeon breast muscle*

0·3 g. muscle, 2·7 ml. phosphate saline, *p*H 7·4; 40°; 1 hr.

Substrate added (final conc.)	μl. succinate found
—	12·6
0·01 *M* α-ketoglutarate	34·2
0·02 *M* α-ketoglutarate	67·8
0·01 *M* fumarate	40·8
0·02 *M* fumarate	115

Experiments on heart, kidney, testis and brain

In tissues in which the citric acid cycle occurs, citrate or α-ketoglutarate should appear among the end-products when oxaloacetate is anaerobically metabolized. The examination of the products of the anaerobic metabolism of oxaloacetate therefore offers an approach to the problem as to whether or not the citric acid cycle may occur in any given tissue. With this in view we have examined the anaerobic fate of oxaloacetate in various organs. Typical experiments are recorded in Table 4. The main results are very similar to those

Table 4. *Fate of oxaloacetate in various tissues under anaerobic conditions*

Minced tissue suspended in phosphate saline. Period of incubation 60 min. Quantities in μl.

Tissue ...	Sheep testis		Guinea-pig kidney		Sheep heart		Sheep brain	
Conc. of suspension	1 part tissue + 2 parts medium		1 part tissue + 9 parts medium		1 part tissue + 9 parts medium		1 part tissue + 6·5 parts medium	
Quantity of suspension	5 ml.		4 ml.		4 ml.		4 ml.	
	Control	Oxaloacetate	Control	Oxaloacetate	Control	Oxaloacetate	Control	Oxaloacetate
Observed:								
Oxaloacetate added	0	2240	0	1792	0	1792	0	1792
Pyruvate found	0	1308	0	400	0	290	0	1120
CO_2	—	—	—	—	160	1478	—	—
Succinate	15	18	54	12	16	42	0	0
α-Ketoglutarate	206	384	72	284	94	308	44·5	148·5
Fumarate + malate	122	455	110	1210	96	1048	0	213
Citrate	22	134	10	64	0	58	0	19·5
Lactate	257	349	—	—	—	—	112	190
Calculated:								
Oxaloacetate metabolized	932		1392		1502		672	
Yield of succinate	0·3%		3·0%		1·7%		0	
Yield of α-ketoglutarate	19·1%		15·2%		14·2%		15·5%	
Yield of fumarate + malate	35·7%		79·0%		63·4%		31·6%	
Yield of citrate	12·0%		3·9%		5·8%		2·9%	
Yield of lactate	9·9%		—		—		11·6%	
Yield of CO_2	—		—		68·4%		—	

obtained with pigeon breast muscle. All the tissues examined form α-ketoglutarate in amounts of the expected order of magnitude. In brain and testis the decomposition of oxaloacetate into pyruvate is greater than in the other tissues. This may be due to the fact that the rates of metabolism are lower in these tissues.

The formation of α-ketoglutarate was to be expected in heart and kidney, since it was already known that the citric acid cycle occurs in these tissues [Breusch, 1939; Smyth, 1940]. For brain and testis the result is somewhat unexpected since several observations seemed to suggest that here the citric acid cycle does not occur [see Banga *et al.* 1939]. Although the formation of α-ketoglutarate from oxaloacetate cannot be regarded as final proof of the occurrence of the citric acid cycle (there might be an alternative pathway from oxaloacetate to α-ketoglutarate) it becomes highly probable that the citric acid cycle, or at least some of its component reactions, plays a part in the metabolism of brain and testis.

The data published by Evans [1940] show that oxaloacetate reacts in pigeon liver in the same way as in pigeon breast muscle.

SUMMARY

In pigeon breast muscle, sheep heart and guinea-pig kidney, added oxaloacetate yields CO_2, fumarate, malate, α-ketoglutarate, citrate, succinate and pyruvate (when the conditions are anaerobic). These substances account for all the added oxaloacetate. The quantities formed are in agreement with the citric acid cycle theory.

Sheep testis and sheep brain also form citrate and α-ketoglutarate from oxaloacetate and this in our opinion suggests that the citric acid cycle, or at least some of its component reactions, can occur in these tissues. It is pointed out, however, that it is not yet possible to assess the quantitative significance of these reactions in the normal metabolism of brain and testis.

This work was aided by grants from the Rockefeller Foundation and the Medical Research Council.

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147. THE EFFECT OF ELECTROLYTES ON THE RESPIRATION OF PIGEON BREAST MUSCLE

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(*Received 1 July 1940*)

It is known from the work of Stare & Baumann [1936], Greville [1937], Elliott & Elliott [1939], Elsden [1939], and Krebs & Eggleston [1940], that minced animal tissues respire best in a medium containing Na, K, Mg, Cl and PO_4 ions, but no Ca ions. A medium used in this laboratory, in which the above ions are present in approximately optimal concentrations, contains 0·158 *M* Na, 0·00452 *M* K, 0·00113 *M* Mg, 0·122 *M* Cl, and 0·022 *M* PO_4.

In experiments in which the effect of varying concentrations of Mg on the respiration of muscle tissue was studied, it was found that the optimal concentration of Mg is not constant, but depends on other factors, especially on the concentration of K. Furthermore the optimal concentration of K is not fixed; it is lowered by Mg.

Methods

The manometric experiments were carried out in conical cups. Unless otherwise stated, 3 ml. of a muscle suspension, prepared from 4 g. minced pigeon breast muscle and 45 ml. medium, were measured into the cup and the volume brought to 4 ml. by addition of substrate, electrolytes and water. Fumarate was used as a substrate. The final suspension in the cup always contained 1 part of muscle in 15 parts of medium. The centre cup contained 0·2 ml. 2 *N* NaOH. The manometers were filled with O_2 and the O_2 uptake was measured at 40° after an equilibration period of 10 min.

It should be noted that the "phosphate buffer" was prepared from Na_2HPO_4 and HCl, and therefore contained 0·02 *M* NaCl. "Phosphate saline" was the medium described by Krebs & Eggleston [1940]. These authors also give further details regarding the preparation of the suspension and the composition of media.

Effect of potassium

The effect of potassium on the respiration is shown in Table 1 and Fig. 1. K causes a 40–50% increase in respiration, the optimal concentration being 0·04 *M*. This effect of K is especially marked in the later stages of the experiment, as shown in Fig. 1.

Effect of magnesium

The effect of Mg, when added to muscle suspension in phosphate buffer, is shown by Tables 2 and 3.

Mg (0·0125 *M*) activates the respiration of pigeon breast muscle in phosphate buffer 110%. This confirms the results of Greville [1937].

Further experiments show that the optimum Mg concentrations depend on the concentration of other ions in the medium. In a medium containing Na, K, and Cl in "physiological" concentrations, the optimal Mg concentration is

Table 1. *The effect of K on muscle respiration*

1 ml. stock suspension (4 g. tissue in 15 ml. phosphate buffer), 0·1 ml. 0·2 *M* fumarate, 2 ml. salt solution, volume adjusted to 4 ml. with H_2O. For comparison the O_2 uptake of 3 ml. muscle suspension in phosphate saline was measured (cup 7).

Cup	Salt solution added to 1 ml. stock suspension (except cup 7)	Final conc. of K *M*	μl. O_2 uptake in 120 min.	% increase due to K
1	2·0 ml. 0·9% NaCl	0	746	0
2	1·5 ml. 0·9% NaCl, 0·5 ml. 1·15% KCl	0·0192	1053	41
3	1·0 ml. 0·9% NaCl, 1·0 ml. 1·15% KCl	0·0385	1141	53
4	0·5 ml. 0·9% NaCl, 1·5 ml. 1·15% KCl	0·0578	1074	44
5	2·0 ml. 1·15% KCl	0·077	1076	44
6	1·9 ml. 0·9% NaCl, 0·08 ml. 1·15% KCl 0·02 ml. 3·84% $MgSO_4$	0·0031	1277	—
7	—	—	1865	—

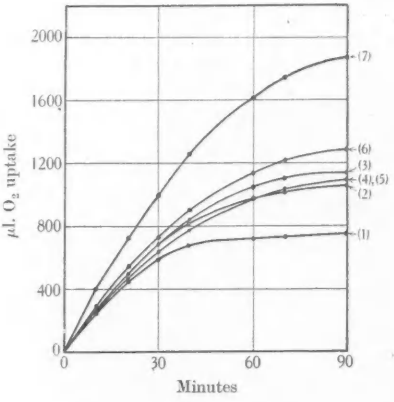

Fig. 1.

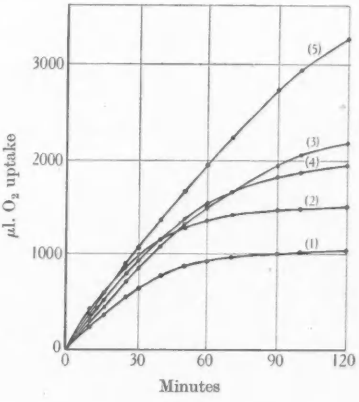

Fig. 2.

Fig. 1. The effect of K on muscle respiration. The numbers of the curves correspond with the numbers of the cups in Table 1.

Fig. 2. The effects of Mg and Mn on muscle respiration. The numbers of the curves correspond with the numbers of the cups in Table 2.

Table 2. *The effects of Mg and Mn on muscle respiration*

3 ml. muscle suspension in phosphate buffer (except in cup 5), 0·1 ml. 0·2 *M* fumarate, electrolytes, H_2O up to 4 ml.

Cup	Added electrolyte	Final conc. of added electrolyte *M*	μl. O_2 uptake in 120 min.	% increase due to addition
1	0	0	1024	0
2	0·3 ml. 0·01 *M* $MnSO_4$	0·00075	1487	45
3	0·5 ml. 0·1 *M* $MgCl_2$	0·0125	2157	110
4	0·3 ml. 0·01 *M* $MnSO_4$ 0·5 ml. 0·1 *M* $MgCl_2$	0·00075 0·0125	1919	87
5	3 ml. muscle suspension in phosphate saline	—	3247	217

0·0025 *M* (Table 4). If the K concentration is changed to 0·0385 *M*, the optimal Mg concentration is about 0·00125 *M* (Table 5). Addition of Mg to the phosphate saline has no significant effect (Table 6).

Table 3. *Optimal concentration of Mg in phosphate buffer*

3 ml. muscle suspension in phosphate buffer, electrolyte, 0·1 ml. 0·2 *M* fumarate, volume made up to 4 ml. with phosphate buffer.

Cup	Added electrolyte	Final conc. of Mg *M*	μl. O_2 uptake in 120 min.	% increase due to Mg
1	0	0	606	0
2	0·1 ml. 0·1 *M* $MgCl_2$	0·0025	1432	136
3	0·2 ml. 0·1 *M* $MgCl_2$	0·005	1743	188
4	0·4 ml. 0·1 *M* $MgCl_2$	0·01	1932	219
5	0·8 ml. 0·1 *M* $MgCl_2$	0·02	1788	195

Table 4. *Optimal concentration of Mg in a medium containing Na, K, Cl and PO_4 in physiological concentrations*

3 ml. muscle suspension in a solution containing 100 parts 0·9% NaCl, 4 parts 1·15% KCl, 30 parts phosphate buffer. 0·1 ml. 0·2 *M* fumarate, electrolyte, volume made up to 4 ml. with H_2O.

Cup	Added electrolyte	Final conc. of Mg *M*	μl. O_2 uptake in 120 min.	% increase due to added Mg
1	0	0	856	0
2	0·05 ml. 0·1 *M* $MgCl_2$	0·00125	1332	56
3	0·1 ml. 0·1 *M* $MgCl_2$	0·0025	1622	89
4	0·2 ml. 0·1 *M* $MgCl_2$	0·005	1450	69
5	0·4 ml. 0·1 *M* $MgCl_2$	0·01	1112	30

Table 5. *Optimal concentration of Mg in a medium containing 0·0385 M K*

1 ml. stock suspension (2 g. minced muscle in 7·5 ml. phosphate buffer). 0·1 ml. 0·2 *M* fumarate, 2 ml. salt solution containing equal parts of 0·9% NaCl and 1·15% KCl, electrolyte, volume made up to 4 ml. with phosphate buffer.

Cup	Added electrolyte	Final conc. of Mg *M*	μl. O_2 uptake in 90 min.	% increase due to Mg
1	0	0	1201	0
2	0·1 ml. 0·025 *M* $MgCl_2$	0·000625	1394	16
3	0·2 ml. 0·025 *M* $MgCl_2$	0·00125	1566	30
4	0·1 ml. 0·1 *M* $MgCl_2$	0·0025	1515	26
5	0·2 ml. 0·1 *M* $MgCl_2$	0·005	1517	26

Table 6. *Effect of Mg added to muscle suspended in phosphate saline*

3 ml. suspension in phosphate saline, 0·1 ml. 0·2 *M* fumarate, electrolyte, volume brought to 4 ml. with H_2O.

Cup	Added electrolyte	Final conc. of Mg *M*	μl. O_2 uptake in 120 min.	% change due to added Mg
1	0	0·00085	1521	0
2	0·05 ml. 0·05 *M* $MgCl_2$	0·00147	1701	+12
3	0·1 ml. 0·05 *M* $MgCl_2$	0·0021	1666	+ 9
4	0·2 ml. 0·05 *M* $MgCl_2$	0·00336	1302	−14·5
5	0·4 ml. 0·05 *M* $MgCl_2$	0·00586	1384	− 9

Effect of manganese

It is known from the work of Negelein (quoted by Warburg & Christian [1936]), Ohlmeyer & Ochoa [1937], and Adler *et al.* [1939], that Mg plays an important part in cell metabolism, which in some cases resembles that of Mn.

As shown by Table 2 and Fig. 2, Mn in low concentrations ($0{\cdot}75 \times 10^{-3}\,M$) has an activating effect on the respiration of minced muscle suspended in phosphate buffer. This effect is limited to the first 30 min., and it is not maintained afterwards. Mn thus cannot replace Mg.

Mn has no activating effect when added to muscle suspensions in phosphate saline. In the later periods of the experiment there is an inhibition of the O_2 uptake. The Mn effect on muscle suspensions in phosphate saline does not depend on the substrate added. The following substrates have been tested: fumarate, pyruvate, citrate, α-ketoglutarate, glutamate and boiled muscle extract.

SUMMARY

The O_2 uptake of minced pigeon breast muscle suspended in various saline media was measured.

The optimal K concentration was found to be $0{\cdot}0385\,M$ if the medium contained $0{\cdot}02\,M$ phosphate and $0{\cdot}0425\,M$ NaCl. The optimal K concentration was much lower ($0{\cdot}0034\,M$) when the medium contained $0{\cdot}02\,M$ phosphate, $0{\cdot}092\,M$ NaCl and $0{\cdot}00085\,M$ $MgSO_4$.

The optimal Mg concentration depends on the concentration of K and may vary between 0·00085 and $0{\cdot}01\,M$. Mg and K can partially, but not completely, replace one another.

Mn increases the O_2 uptake of muscle suspended in phosphate buffer, but does not replace Mg.

The author wishes to express his gratitude to Dr H. A. Krebs for kind help and advice.

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148. THE DETERMINATION OF MINUTE AMOUNTS OF LEAD IN URINE

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(*Received 25 July 1940*)

For a comparative study of lead and porphyrin metabolism in lead workers a rapid and accurate method for the determination of urinary lead was necessary. Ross & Lucas [1935] separated lead by entrainment with calcium oxalate and subsequent colorimetric estimation with dithizone, as described by Fischer [1930]. Tompsett & Anderson [1935; 1939] have extracted lead from biological materials as a complex either with sodium diethyldithiocarbamate or with dithizone, both substances being stable in the presence of ammoniacal cyanide. Hubbard [1937] prefers to use dithizone throughout for extraction and analysis; modifications have been introduced [cf. Bambach, 1939], such as the use of hydroxylamine hydrochloride to prevent the oxidation of dithizone in the initial extraction.

Employing Tompsett's method, the writer experienced losses during the recovery of lead from urine owing to its entrainment with phosphate and finely divided silica. Unavoidable excess of perchloric acid often led to the formation of a yellow oxidation product from dithizone. Digestion with deleaded H_2SO_4 to destroy organic material and the use of perhydrol overcame these difficulties. Lead was quantitatively removed from the H_2SO_4 digest as the complex with sodium diethyldithiocarbamate in ether. 100 ml. urine required only 5 ml. pure H_2SO_4 and approximately the same volume of ammonia (sp. gr. 0·880), both rendered lead-free. The blank was 3·4 μg. The extracted lead was digested with 1 ml. H_2SO, with a final addition of 4 or 5 drops of perhydrol. Excess of perhydrol was easily removed and no trouble was experienced from the oxidation of dithizone. For the final comparison as dithizonate the technique of King *et al.* [1937] was used with spectrum green filter 604 and neutral grey screen density 0·74 as standard.

Procedure

Pyrex glassware and glass-distilled water were used throughout. Reagents were purified as follows.

2*N* H_2SO_4 was twice shaken with CCl_4-ether mixture (1:20) and 20 mg. Na diethyldithiocarbamate and run off. The acid was then shaken with ether alone and the latter removed by careful warming on the water bath.

20% Na citrate and ammonia were deleaded in a similar manner.

Merck's perhydrol and 10% KCN were obtained lead-free.

5 mg. dithizone dissolved in 5 ml. CCl_4 were shaken with 5% NH_4OH. The aqueous layer was acidified with *N* HCl and the dye taken up in CCl_4, leaving traces of lead in the aqueous phase. Finally, the purified dithizone was extracted with 5% NH_3 just before use.

100 ml. urine were evaporated with 100 ml. 2*N* H_2SO_4 in a 350 ml. Kjeldahl flask and digested with the addition of 4 drops of perhydrol until clear. The digest was diluted with water, 5 ml. 20 % Na citrate were added and the solution was made alkaline to litmus with ammonia (sp. gr. 0·880). After the addition of 5 ml. 10 % KCN, lead was removed by shaking twice with ether and approximately 20 mg. Na diethyldithiocarbamate. The ethereal extract was washed with water, the solvent evaporated and the residue digested with 1 ml. H_2SO_4. 3–4 drops of perhydrol were added and heating was continued for 5 min. after clearing.

The acid was diluted with 4 ml. water and 0·2 ml. CH_3COOH; 3 ml. ammonia (sp. gr. 0·880) were added to make the reaction alkaline. This solution was washed out into a 50 ml. separating funnel (with very short stem) with 5 ml. 1 % KCN, and 5 ml. CCl_4 were added. After vigorous shaking with slight excess of dithizone solution (2–3 drops) the aqueous phase was drawn off with a Pasteur pipette and the remaining dithizone washed out with 1 % KCN. The coloured CCl_4 layer was run off and compared with a standard (lead acetate) in a Klett microcolorimeter, using photometric technique.

Discussion

Known quantities of lead covering a wide range were added to normal urine to test the accuracy of the method. The data are presented in Table 1 and it is evident that the recovered amounts agree to within 3 μg. with the amounts added.

Table 1. *Recovery of lead added to normal urine*

Vol. of urine ml.	Amount of lead added μg.	Total lead recovered μg.	Reagent blank μg.	Total lead corrected for blank μg.	Amount of added lead recovered μg.
50	0	5·6	3·4	2·2	—
	10	15·6		12·2	10·0
	20	25·7		22·3	20·1
	30	33·8		30·4	28·2
	40	46·9		43·5	41·3
100	0	11·2	3·4	7·8	—
	20	33·1		29·7	21·9
	30	43·6		40·2	32·4
	40	52·6		49·2	41·4
100	0	11·1	3·4	7·7	—
	20	33·6		27·7	20·0
		28·6			
	30	44·1		40·7	33·0
	40	53·6		50·2	42·5

The method was now applied to the determination of the lead excretion in normal adult urine and urine from lead workers. For a series of 10 normal adults with no known exposure to lead the figures varied from 0·038 to 0·080 mg./l. with an average of 0·057 mg./l. These values are in close agreement with those reported by Kehoe *et al.* [1935] and by Ross & Lucas [1935].

Samples of urine from lead workers exposed to materials containing negligible amounts of tin, antimony and bismuth were collected throughout working hours. No bismuth could be detected in the samples by extracting with dithizone at *p*H 2 after treatment with 1 % HNO_3. 120 determinations were made over a period of several months and representative results are shown in Table 2. The urinary excretion of this group of lead workers varied from 0·1 to 0·35 mg./l., and 100 ml. duplicates agreed within 0·004 mg.

Table 2. *Urinary lead excretion in lead workers*

Urine no.	Lead recovered from 100 ml. urine μg.	Corrected reading for 100 ml. urine μg.
1	28·0	24·6
3	36·8	33·4
	37·9	34·5
5	25·0	21·6
	29·1	25·7
7	17·6	14·2
11	16·2	12·8
	15·5	12·1
13	18·8	15·4
	20·0	16·6
	19·4	16·0
17	17·8	14·4
	18·4	15·0
22	16·3	12·9
	17·0	13·6
23	25·4	22·1
27	17·7	14·3
41	22·2	18·8
51	13·7	10·3
86	25·4	22·0
112	24·3	10·9
114	19·0	15·6

SUMMARY

A method is described for the determination of lead in urine. 50–100 ml. samples are adequate and the method is economical in both time and reagents.

I wish to thank Dr J. F. Wilkinson for his interest in this work, and Dr R. E. Lane of the Chloride Electrical Storage Co., Ltd., for his co-operation.

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149. THE SERUM PROTEINS IN MULTIPLE MYELOMATOSIS

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(*Received 31 July 1940*)

THE serum of patients suffering from multiple myelomatosis has for a considerable time been known to show gross divergences in constitution in comparison with normal human serum. Among these abnormalities may be mentioned a marked hyperproteinemia associated with a change in the albumin-globulin ratio. The condition is also frequently, but not invariably, associated with Bence-Jones proteinuria. An exhaustive review of the literature has recently been given by Bonsdorff *et al.* [1938].

The data on two such sera [McFarlane, 1935] encouraged a further extensive study by the ultracentrifugal and recently improved electrophoretic techniques. This paper is a report of the results obtained in the examination of the sera from five cases by these methods.

Whilst this work was in progress two papers appeared [Jersild & Pedersen, 1938; Longsworth *et al.* 1939], the first giving ultracentrifugal, the second electrophoretic data on the serum of multiple myelomatosis. These will be referred to again in the discussion.

EXPERIMENTAL

The methods used in the treatment and examination of the sera followed those adopted in the examination of a series of normal human sera [Kekwick, 1939].

The sera were dialysed undiluted at constant volume against phosphate buffer *p*H 8·0, $\mu = 0{\cdot}1$, until ionic equilibrium was attained. Any slight precipitate which formed was centrifuged off, and the refraction due to the non-dialysable materials present determined with the dipping refractometer ($\lambda = 546$ mμ). These data are given in Table 2, column 2, headed "*n* Protein", and represent the differences in the refractive indices, n_1, and n_0 of the protein solution and buffer respectively. For examination the dialysed sera were diluted with buffer to $n_1 - n_0 = 0{\cdot}00300$.

The ultracentrifugal examinations were made in a field strength of 270,000 times gravity, with optical observations by the diagonal Schlieren method [Philpot, 1938]. The light source was a high-pressure mercury arc from which monochromatic light, $\lambda = 546$ mμ, was isolated by a suitable filter.

By optical projection of the recorded photographs, with an enlargement of eight diameters on to millimeter graph paper, the boundary positions were measured for sedimentation constant calculations. Tracings were also made, with the same magnification, for the analytical determinations of the quantities of components present in the sera. The sedimentation constants (S_{20}) quoted were reduced to water at 20° according to the usual method [Svedberg & Pedersen, 1940].

All the electrophoretic measurements were made in the Tiselius [1937] apparatus at 0°. For the analytical experiments a potential gradient of 5 V./cm. was applied, higher gradients up to 10 V./cm. being used in mobility experiments.

The Lamm scale method, in conjunction with the Toepler Schlieren method, was used for the electrophoretic analysis. A sodium vapour lamp illuminated the U-tube, and the migration was recorded photographically. Further details of treatment were the same as in a previous paper [Kekwick, 1939].

For electrophoretic mobility measurements on proteins isolated from the sera, the Toepler Schlieren method was used at first, but later the diagonal Schlieren method was substituted. In these experiments a protein concentration corresponding to $n_1 - n_0 = 0{\cdot}00150$ was employed to reduce any concentration effect on mobility as far as possible, whilst still enabling a well-defined boundary to be observed over long periods. After a suitable time interval in the experiments, the current was reversed and the boundaries brought back to the starting position. The mobilities given are mean values calculated from the ascending and descending boundaries, and have been corrected for the viscosity effects of the buffer salts.

As a further means of characterizing the proteins isolated from the sera, carbohydrate-nitrogen ratios were determined. The carbohydrate determinations were made by the method of Sørensen & Haugaard [1933], mannose being used as an arbitrary standard. In earlier determinations a Zeiss step photometer was employed in the measurements of the extinctions. Later a photoelectric photometer was utilized, the blue line (436 mμ) of the mercury arc being found very suitable for the absorption measurements. Nitrogen estimations were made by the micro-Kjeldahl method using selenium oxychloride as a catalyst.

The hydrogen electrode was used for *p*H measurements, with *N*/10 HCl (*p*H 1·075) for standardization.

I. Results of ultracentrifugal and electrophoretic examination of the sera

The data obtained from the examination of the sera, which have been arranged in descending order of protein content except for serum 5, are collected in Tables 1 and 2.

In the ultracentrifuge sera 1–4 showed two components corresponding, as an examination of the sedimentation constants in Table 1 shows, to the albumin and globulin of normal human serum. The slight differences in the sedimentation constant values for the pathological and the two normal human sera, are probably due to differences in the amounts of the components in the two types of sera. Serum 5, which is very complex, showed five components, two corresponding in sedimentation constant to the components of normal human serum, and three of higher values not found in normal human serum. Samples of albumin and total globulin were separated electrophoretically from this serum. The albumin sedimented as a single component $S_{20} = 4{\cdot}15$, the globulin showing four components, $S_{20} = 6{\cdot}54$, 9·26, 11·3 and 13·3.

In Fig. 1 are shown sedimentation diagrams of normal human serum and myelomatosis sera 3 and 5, each obtained 45 min. after reaching full speed.

All the myelomatosis sera show an increase in the proportion of globulin in comparison with normal human serum. The analytical data in Table 2, which give the percentages of the total refraction due to each component, refer in each case to the mean value obtained from two ultracentrifuge exposures. It is apparent that the proportion of globulin increases with the total protein content of the serum.

The electrophoretic examination of these sera revealed four components, albumin, α, β, and γ globulins. In most cases the data (Table 2) are mean values

Table 1. *Sedimentation constants S_{20} of human serum components*

Serum	Albumin	Globulin
Myeloma 1	4·29	6·34
Myeloma 2	4·12	6·22
Myeloma 3	4·06	6·54
Myeloma 4	4·10	6·39
Myeloma 5	4·32	6·59, 9·09, 11·2, 14·3
Normal 1	3·86	6·06
Normal 5	3·79	6·01

Phosphate buffer *p*H 8, $\mu = 0{\cdot}1$. Concentration $n_1 - n_0 = 0{\cdot}00300$. The sedimentation constants, S_{20}, are in units of 10^{-13}.

Table 2. *Ultracentrifugal and electrophoretic analysis of human serum components*

		Ultracentrifugal		Electrophoretic				
					Globulins			
Serum	*n* Protein	Albumin	Globulin	Albumin	α	β	γ	Albumin
1. (J. B.)	0·02220	31·2	68·8	16·0	2·8	4·2	76·9	0·00355
2. (C. E.)	0·02120	41·2	59·0	22·4	2·9	9·7	65·0	0·00475
3. (L. G.)	0·01941	44·9	55·0	30·4	3·2	11·2	55·3	0·00590
4. (S. H.)	0·01494	67·0	33·2	45·7		14·5	39·8	0·00683
5. (J. J.)	0·02441	30·3	69·7	18·6	15·0	61·7	4·7	0·00454
Normal	0·01293	78·0	22·0	62·5	5·0	11·5	21·0	0·00808
Normal*	—	—	—	64·2	8·5	16·8	10·5	—
Normal†	—	—	—	66·2	7·9	13·9	13·2	—

Phosphate buffer *p*H 8, $\mu = 0{\cdot}1$. Concentration $n_1 - n_0 = 0{\cdot}00300$.
The amounts of components are percentages of the total refractive increment.
* Svensson [1939]. † Longsworth *et al.* [1939].

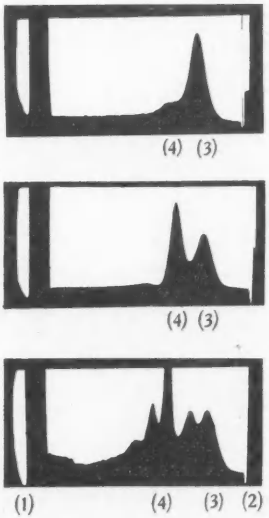

Fig. 1. Sedimentation diagrams. Top: normal human serum. Centre: myelomatosis serum 3. Bottom: myelomatosis serum 5. Phosphate *p*H 8, $\mu = 0{\cdot}1$, $n_1 - n_0 = 0{\cdot}00300$. Field strength 270,000 × gravity. All photographs taken 45 min. after reaching full speed. (1) Cell index. (2) Meniscus. (3) Albumin. (4) Globulin.

from four exposures, two from the anode and two from the cathode limb. Owing to the quantitative distribution of the components it was possible in these cases to correct for the δ boundary on the anode side, and to make complete analysis of the cathode limb curves, neither of which procedures was possible for normal human serum under the experimental conditions necessarily adopted.

Figs. 2 and 3 are diagrams of sera 3 and 5 respectively, taken 2 hr. after starting the current.

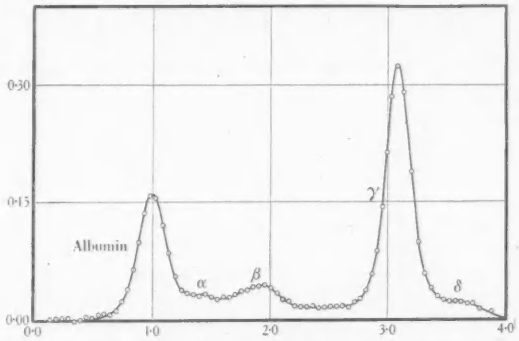

Fig. 2. Electrophoresis diagram. Myelomatosis serum 3. Phosphate *p*H 8, $\mu = 0{\cdot}1$, $n_1 - n_0 = 0{\cdot}00300$, 5 V./cm. Exposure 120 min. after starting current. Abscissa: distance in U-tube in cm. Ordinate: scale line displacement in mm. Anode limbs.

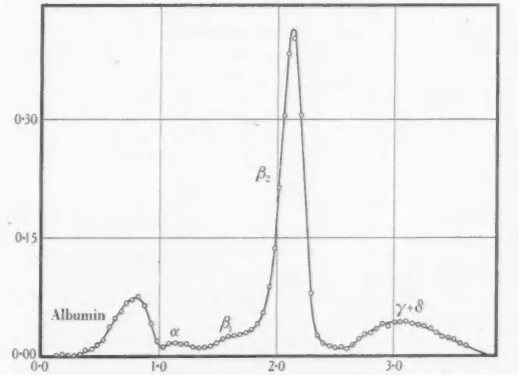

Fig. 3. Electrophoresis diagram. Myelomatosis serum 5. Phosphate *p*H 8, $\mu = 0{\cdot}1$, $n_1 - n_0 = 0{\cdot}00300$, 5 V./cm. Exposure 120 min. after starting. Abscissa: distance in U-tube in cm. Ordinate: scale line displacement in mm. Anode limbs.

The outstanding characteristics of sera 1–4 are the reduced percentage of albumin and the greatly increased percentage of γ globulin, the deviation from the normal in each case increasing with the total protein content of the serum. The percentage of α and β globulins remains fairly close to that of normal human serum. In contrast with these, serum 5 showed a great increase in β globulin, the percentage of γ globulin being below that found in normal human serum.

With improved optical methods, Svensson [1939] has shown that the β component of normal human serum is in effect composed of two substances whose mobilities lie very close to one another. These may be referred to as β_1 and β_2 in decreasing order of mobility. A similar phenomenon has been observed by the writer in normal and immune horse sera. It would appear that in serum 5 it may be the β_2 component which is increased in amount. The high value for the proportion of α globulin found for this serum is due to the inclusion of the area due to globulin β_1 with it, a finer analysis not being possible. It was not possible to analyse the serum 4 diagrams for α and β globulins separately.

In the last column of Table 2 the refractive increment due to the albumin in the serum, obtained from columns 2 and 5, is given. It appears that not only does the percentage of serum albumin decrease as the protein content of the serum rises, but the absolute concentration also decreases. As data on blood volume were not available, calculations of the total circulating serum albumin could not be made. However, it seems improbable that a doubling of the blood volume could occur, which would be necessary in some of the cases, in order that the total circulating serum albumin should remain constant.

An interpolation is necessary here concerning the values quoted for the amounts of the components determined by electrophoretic analysis of normal human serum.

It has been pointed out [Longsworth *et al.* 1939] that analytical values for components determined from descending limb photographs, in this case the cathode, are likely to be the more accurate owing to the absence of the δ boundary, the ϵ boundary effect being very slight. In the study of normal human serum [Kekwick, 1939] only the percentages of albumin and total globulin were estimated from descending limb exposures, as it was considered that the extrapolation of the curves obtained for the individual globulins was too uncertain to be of value. Ascending limb exposures which apart from the γ and δ components were capable of complete analysis, gave a smaller percentage of albumin than those from the descending limb, and the value for γ globulin was too high owing to the inclusion with it of the effect due to the δ boundary.

Accordingly the ascending limb values have been corrected for the δ boundary effect by multiplication by the ratio

$$\frac{\%\text{ albumin (descending limb)}}{\%\text{ albumin (ascending limb)}},$$

and the total excess percentage subtracted from the γ globulin percentage. The corrected values, which are given in Table 2, represent the means from six normal human sera. Some deviation occurs from the data of other workers.

II. The isolation and characterization of some proteins from the sera

It was felt that an important point to establish was whether the γ globulin, present in such excessive amounts in sera 1–4, had the same characteristics as the γ globulin of normal human serum. The measurement of the mobility of the protein in the analytical experiments at a single pH, and the comparison of this value with the corresponding value for normal human γ globulin, the latter determined in the presence of the other normal serum protein components, was not considered sufficient evidence on which to base any conclusions. Experiments showed that the γ globulin from myelomatosis serum could readily be obtained as an homogeneous substance by simple salting out procedures. The β component of serum 5 was isolated in a similar manner.

It has also been found possible to isolate the γ globulin of normal human serum in large quantities by salt fractionation, the product sedimenting and migrating homogeneously.

The various preparations obtained have been characterized by determinations of sedimentation constant, electrophoretic mobility and isoelectric point, and carbohydrate-nitrogen ratio.

Details of preparations of myelomatosis globulins

Preparation 4. As sera 1 and 2 showed a similar constitution, and in view of the small amount of material available, the residues of the sera in phosphate buffer *p*H 8, $\mu=0{\cdot}1$, were combined and precipitated once with half-saturated $(NH_4)_2SO_4$. The precipitate was centrifuged, washed once by suspension in a small amount of half-saturated $(NH_4)_2SO_4$ and recentrifuged.

In the ultracentrifuge a single component only was found, but in phosphate buffer *p*H 7, $\mu=0{\cdot}1$, a small amount of a faster moving component was observed in the electrophoresis apparatus.

Preparation 7. This was obtained from serum 3 in phosphate buffer *p*H 8, $\mu=0{\cdot}1$. The globulin was precipitated by addition of Na_2SO_4 up to 16 g./100 ml. The precipitate was redissolved in phosphate buffer *p*H 8, $\mu=0{\cdot}1$, and reprecipitated by bringing the concentration of Na_2SO_4 up to 20 g./100 ml. The product sedimented and migrated as a single component.

Preparation 6. By precipitating myeloma serum 5 in phosphate buffer *p*H 8, $\mu=0{\cdot}1$, with 20 g./100 ml. of Na_2SO_4, a quite distinctive product was obtained. The material thrown out of solution rose to the surface and had a sticky consistency. Electrophoretically the material was homogeneous, but under all conditions of concentration and of buffer salt composition four components were observed in the ultracentrifuge.

The isolation of normal human γ globulin

For comparison with the proteins isolated from the various myelomatosis sera, it was decided to isolate normal human γ globulin. For this purpose a salt fractionation technique was elaborated, using the electrophoretic method to control the fractionation.

Addition of 18 g./100 ml. of Na_2SO_4 to normal human serum precipitates the globulins almost completely, the precipitate being only slightly contaminated with serum albumin. If this globulin is dissolved in phosphate buffer *p*H 8, $\mu=0{\cdot}1$, to a volume 40 % that of the initial serum, and the Na_2SO_4 concentration brought up to 12 g./100 ml. a characteristic precipitate forms. Very marked streaming occurs in the solution and tactoid bodies can be observed under the microscope. The precipitate settles rapidly and forms a continuous stickly layer over the bottom of the containing vessel. This material consists of γ globulin contaminated with about 5 % of α and β globulins. Resolution of this precipitate in phosphate *p*H 8, $\mu=0{\cdot}1$, to 2 % of the initial serum volume and reprecipitation with 12 g./100 ml. Na_2SO_4 gives pure γ globulin. The yield of thrice precipitated material is about 7 % of the total initial serum protein.

In Tables 3–6 the data obtained from the examination of the various globulin preparations are presented.

The solutions used in the sedimentation constant determinations all contained $0{\cdot}1M$ NaCl in addition to the buffer salts, to inhibit charge effects. The total ionic strength of the solutions varied between $\mu=0{\cdot}12$ and $\mu=0{\cdot}2$, but the protein content was maintained at $n_1-n_0=0{\cdot}00150$.

Table 3. *Sedimentation constants S_{20} of human serum globulin preparations*

Preparation	*p*H ... 5·1	6·0	7·0	8·0	S_{20} Mean
Myeloma globulin 4	—	6·58	6·63	6·60	6·60
Myeloma globulin 4	—	6·68	6·88	6·86	6·81
Myeloma globulin 6	—	—	—	6·54, 9·26, 11·3, 13·3	—
Normal human γ globulin	6·78	6·76	6·93	6·66	6·78
Buffer composition	Acetate	Phosphate	Phosphate	Phosphate	—
μ=0·120–0·200	NaCl	NaCl	NaCl	NaCl	—

The sedimentation constants, S_{20}, are in units of 10^{-13}.

Table 4. *Myeloma globulins. Electrophoretic mobilities*

*p*H	Preparation 4	Preparation 7	Preparation 6
5·11	2·85	2·71	2·10
5·84	—	1·18	−0·05
5·95	1·00	—	—
6·45	—	0·70	—
6·46	—	—	−0·42
6·99	—	—	−0·89
7·00	−0·01	—	—
7·96	—	−0·34	−1·32
7·99	—	—	−1·40*
8·03	−0·73	—	—
I.E.P.	*p*H 6·99		*p*H 5·81

* Electrophoretically separated from myeloma serum 5.

Mobilities are in units of cm.^{2} V.$^{-1}$ sec.$^{-1}$ $\times 10^{-5}$.

Buffers: ionic strength μ=0·1. All buffers were phosphate mixtures except for *p*H 5·11, which was acetate.

Table 5. *Normal human globulin. Electrophoretic mobilities*

*p*H	Mobility
5·12	2·17
5·94	0·52
6·48	0·01
7·03	−0·27
8·01	−0·88
I.E.P.	*p*H 6·58

Mobilities are in units of cm.^{2} V.$^{-1}$ sec.$^{-1}$ $\times 10^{-5}$.

Buffers: ionic strength μ=0·1. All buffers were phosphate mixtures except for *p*H 5·12, which was acetate.

Table 6. *Carbohydrate-nitrogen ratios for globulin preparations*

Preparation	Carbohydrate-N ratio
Myeloma globulin preparation 4	0·100
Myeloma globulin preparation 7	0·083
Myeloma globulin preparation 6	0·167
Normal human γ globulin	0·077

Globulin preparation 6 was unusual in its sedimentation behaviour, showing four components. Preparations 4 and 7 sedimented as homogeneous substances, though for the former the sedimentation constant is rather low in comparison with that found for normal human γ globulin. For an electrophoretically prepared normal human γ globulin, Kabat [1939] gives $S_{20}=7{\cdot}1\times10^{-13}$, somewhat higher than the mean of $6{\cdot}8\times10^{-13}$ found here for the salted out material.

The mobility data of Tables 4 and 5 are plotted in Fig. 4. From this it is immediately apparent that the globulins isolated from the myelomatosis sera differ in some degree from normal human γ globulin. The mobilities of preparations 4 and 7 are in agreement with one another in the more acid part of the range covered, but show some divergence in the more alkaline region.

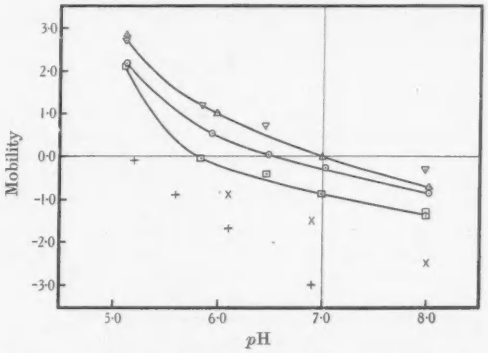

Fig. 4. Mobility-pH curves for human globulin preparations. △ ▽ ⊡ Myeloma globulins 4, 7, 6 respectively. ⊙ Normal human γ globulin. + Normal human β globulin. × Fibrinogen.

Preparation 6, although showing four components in the ultracentrifuge, migrated as a single component at all pH investigated, the mobility curve lying between that of normal human β and γ globulins and differing from that of fibrinogen. The values given for fibrinogen and normal β globulin are those found by Stenhagen [1938] for migration in the presence of all the normal serum components. Some deviations occur in the values for normal human γ globulin determined under his conditions, and those reported here on the pure material. The isoelectric points of the myelomatosis globulin preparations differ markedly from the value found for normal human γ globulin.

A consideration of the carbohydrate-nitrogen ratios of the preparations indicates a similarity between normal human γ globulin and globulins 4 and 7. The fractionation of normal human serum described in the preparation of normal human γ globulin was followed by determinations of carbohydrate-nitrogen ratios as well as by electrophoretic analysis. It became apparent that normal human β globulin had a rather higher carbohydrate-nitrogen ratio of the order 0·14, suggesting that globulin 6 may be more nearly related to normal β than to normal γ globulin. This is in conformity with its electrophoretic behaviour.

Discussion

The myelomatosis sera so far investigated appear to fall into two groups according to their behaviour in the ultracentrifuge. The first group shows the same number of components as normal human serum, the globulin component being quantitatively very much increased. In addition to the sera of this type described in the present communication McFarlane [1935] and Jersild & Pedersen [1938] have each published data on a serum with these characteristics. The second group of sera, of which one is described here, shows several components, in this case five, whilst in one reported on previously by McFarlane [1935] four components were demonstrated.

In the sera of the first group electrophoresis indicated a large increase in γ globulin, whereas in the single second group serum available an excess of β globulin was apparent. It is interesting that of three myelomatosis sera investigated by Longsworth *et al.* [1939] two gave a large increase in β globulin, whilst the third showed no deviation from the normal in appearance.

Those components which appeared in excessive amount in the sera examined here were qualitatively distinguishable from the components of normal human serum, particularly by electrophoresis.

Unfortunately none of the cases whose sera have been examined here showed any proteinuria, neither do Longsworth *et al.* [1939] mention this in connexion with any of the cases they investigated. One case described by McFarlane [1935] had Bence-Jones proteinuria, the serum being of the first type. No further light can, as yet, be thrown on any relationship between the serum proteins and Bence-Jones protein.

The remarkable serum described by Bonsdorff *et al.* [1938] in which spontaneous crystallization occurred was not examined by physical methods, though the crystalline globulin which separated was examined in the centrifuge by Pedersen. The sedimentation constant of this material was $S_{20} = 7{\cdot}1 \times 10^{-13}$ though its molecular weight was 200,000, which is higher than that found for normal human serum γ globulin, viz. 176,000. None of the sera described in this paper revealed any tendency to deposit crystals spontaneously.

Summary

An examination of the sera from five cases of myelomatosis suggests that according to their ultracentrifugal and electrophoretic behaviours the sera are of two types. As far as can be determined, this classification is applicable to those myelomatosis sera which have previously been examined by these methods and reported on in the literature.

In addition to quantitative differences in the constitution of myelomatosis sera as compared with normal human serum, qualitative differences are apparent in some of the protein components.

The author wishes to express his thanks to Dr J. Z. Walker, the Royal Infirmary, Edinburgh; Dr R. G. Macfarlane, the British Postgraduate Medical School, London; Dr E. R. Holiday, the London Hospital, for supplying samples of serum.

The author is indebted to the Medical Research Council for personal and research expenses grants.

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APPENDIX

Clinical notes

J. B. Male 27 years. July 1936, knock on forehead followed by swelling. October 1936, swellings in skull and ribs. X-ray: several areas of bone destruction in skull, tumour right lateral forehead, rib destruction with tumour projecting into chest; area of pelvic bone destruction. Palliative treatment to skull and ribs. Died 26. iii. 39. Serum November 1938.

C. E. Male 50 years. September 1937, deformity of upper dorsal spine. X-ray: collapse of L.V. 3 due to myeloma of spine; myelomatous changes in right leg. X-ray therapy to spine. January 1940, other bones showed myelomatous changes. General condition fairly satisfactory. Serum November 1938.

L. G. Female 70 years. May 1938, soft area in scalp behind left ear. X-ray: numerous areas of increased transradiancy throughout skull; right femur and humerus sharply defined defects. Sternal puncture: characteristic of multiple myelomatosis. Serum April 1939.

S. H. Male 61 years. 1936, pain lower sternum. 1937, lump developed on lower sternum. Part of lower sternum removed, "crackled egg shell appearance". 1938, pain in shoulder blades and lower ribs. Deep X-ray treatment. 1939, pain sitting or lying, no spinal tenderness. Sternal puncture: characteristic of multiple myelomatosis. X-ray of skeleton inconclusive. No Bence-Jones proteinuria. Serum May 1939.

J. J. Male 60 years. Upper abdominal pain for 35 years. Sternal puncture: characteristic picture of multiple myelomatosis. X-ray: skull showed numerous small areas of increased translucency. Trace of albumin in urine. No Bence-Jones proteinuria. Died 21. v. 39. P.M., multiple myelomatosis; hypostatic pneumonia. Serum April 1939.

150. THE INACTIVATION OF SOME PLANT VIRUSES BY UREA

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(*Received 30 July 1940*)

Introduction

Of the simple organic substances known to affect proteins in neutral solution, urea has been the most extensively studied. There is no obvious reason for this, for urea is neither a more powerful denaturing agent nor a better protein solvent than some of the others. The fact that it is a normal metabolic product is not really relevant, for most of the effects studied are observed only at 10 or more times the physiological concentration. Detailed studies on the action of urea have been made only in recent years, but some of its more striking effects were early noticed.

Buchner [1874; 1876] reviewed and extended earlier physiological studies on muscle stimulation, and noticed that within a few minutes of putting strips of muscle in concentrated urea solutions there was osmotic shrinking, followed by great swelling, though little of the material went into solution. Limbourg [1887] made similar experiments with frog nerves and seems to have been the first to discover that urea solutions could dissolve some otherwise insoluble proteins. He described the behaviour of such solutions towards commonly used protein precipitants and in 1889 found that solutions of fibrin in urea did not coagulate when heated. Loss of heat coagulation in 10% urea solutions was found with Bence-Jones protein by Magnus-Levy [1900] and with the commoner proteins by Ramsden [1902], who also described graphically the disintegration of a frog in saturated urea solution, and recommended the latter as a histological reagent. Braun [1933] and Stein & Miller [1938] adopted this recommendation for the histological recognition and preparation of elastin, which is one of the few proteins insoluble in concentrated urea solution. The solvent properties of urea have also been used in the preparation of other proteins, e.g. by Cook & Alsberg [1931] for glutenin, by Urban [1936] for a liver globulin mixture, by McKay & Lamson [1936] for pollen proteins and by Kondo *et al.* [1939] for soya bean proteins. Walker [1940] extracted antigens from *Salmonella aertrycke* with urea, but the method is not generally applicable, for Fuller [1938] and Miles & Pirie [1939] found it of no value with other bacteria.

Many workers have found that the presence of urea protects proteins from the precipitating effects of some reagents. Pauli & Rona [1902], Arnd & Hafner [1926], Jirgensons [1936] and Heim [1937] showed that more salt was necessary to get precipitates in solutions of gelatin, euglobulin, haemoglobin and fibrinogen if urea was present. Moser [1927] found serum proteins more difficult to precipitate by dilution or acidification in the presence of urea, and Ogiu & Pauli [1932] stabilized the complex of serum albumin and colloidal gold with urea and with glycine.

Detailed studies of the changes occurring when a protein is dissolved in strong urea solution, and the correlation of these with observations made in the presence of other organic substances, date from Spiro's [1900] work on their effect on coagulation by heat. Ramsden [1913], however, was the first to relate these changes with the group of protein-modifying processes known as denaturation. Although Ramsden's results were published in both English and German [Ramsden & Chavasse, 1913], they attracted little attention, and widespread interest was not aroused until Anson & Mirsky [1929] studied the denaturation of haemoglobin by urea and some other substances. Burk & Greenberg [1930], while continuing their earlier [1928] measurements of the osmotic pressure of proteins in 40% urea solution, described the denaturation of egg albumin and horse haemoglobin, and found that the osmotic pressure of haemoglobin denatured in this way corresponded to a molecular weight of 34,000, i.e. one-half of the original value.

Hopkins [1930] found that denaturation of egg albumin in the presence of urea proceeded more rapidly at 0° than at 37°, an interesting observation confirmed by Ramsden [1930]. Stanley & Lauffer [1939] found that the disintegration of tobacco mosaic virus also proceeded more rapidly at 0° than at 25°, but that it proceeded more rapidly at 40° than at 25°. Whether this increase in denaturation rate at low temperatures applies at all generally to proteins is uncertain, for Diebold & Jühling [1938] found that fibrinogen was rapidly denatured at 37°, whereas at 4° it was more stable in the presence of 23% urea than it was in water. However, the behaviour of fibrinogen seems to depend on its purity, as Diebold [1938] found that it was completely destroyed at 4° if urea was added to unfractionated plasma. Jühling & Wöhlisch [1938], who confirmed the difference between plasma and isolated fibrinogen, suggested that it may result from the activation of a fibrinolytic enzyme normally present in plasma in an inactive form.

1. *Evidence for the attachment of urea to proteins*

Observations similar to those of Buchner [1876] on the swelling of cornea in urea solutions have been made by Stoeltzner [1925] and Stoye [1925] on pieces of dura mater. Lloyd & Mariott [1933] measured the swelling of silk fibres in urea solution and found that there was a weakening of the lateral binding forces of the fibres. They suggested that urea was absorbed on the internal surfaces of the fibres, a conclusion agreeing well with the observation of Trogus & Hess [1933] that silk fibroin, after soaking in 50% urea, gave an X-ray pattern showing a regular arrangement of urea within the fibre. On the other hand, Astbury *et al.* [1935] do not mention such a pattern in washed films of denatured edestin or egg albumin prepared from urea solutions. Worschitz & Herman [1934] found that the micelle structure of muscle fibres disappeared in urea solutions and did not reappear when the urea was washed out. The phenomena of the swelling of crystalline or fibrous structures with urea and other substances were discussed by Katz [1932] and by Lloyd & Shore [1938]. Insoluble proteins that are not fibrous have been little studied, although Fischer & Sykes [1915] published some observations on the swelling of blocks of gelatin in solutions of urea too dilute to cause solution.

Attempts to use urea to measure the "bound water" in proteins have given further evidence that the first step in the action is the binding of urea to the protein. For example, Oda [1930] found that 5% of the water in serum was not available for dissolving glucose, whereas it was all available for the solution of urea. Similarly, Versmold [1931] measured the freezing points of solutions of

egg albumin containing either glucose, glycerol or urea and found that much more of the urea was bound to the protein. Finally, measurements of the optical rotatory power of proteins in different solvents put urea in a special category. Dill & Alsberg [1925] found that gliadin had a higher rotation in 30% urea than in various dilutions of ethyl and propyl alcohols, and Pauli & Weiss [1931] found that proteins in solutions of sodium benzoate and salicylate, which like urea are denaturing agents, had the same rotation as in water, whereas in urea solutions they had a higher rotation.

2. *The size and shape of proteins in urea solution*

Solution in urea seems to affect the osmotic pressure of individual proteins differently. Burk & Greenberg [1930] found that in urea solution horse haemoglobin had half its normal molecular weight, as measured by osmotic pressure, and edestin one-quarter. Burk [1937, 1] found similar changes in osmotic pressure with amandin and excelsin, and [1940] *Limulus* haemocyanin. Wu & Yang [1932] confirmed the fall in the molecular weights of ox and horse haemoglobins but found the values for sheep and dog haemoglobins to remain unchanged after treatment with urea, although Drabkin [1939] has shown that this treatment does denature dog haemoglobin. Hand [1935] claimed that the effects of hydration had been overlooked in these measurements on haemoglobins, but his criticism met with little support [cf. Steinhardt, 1938]. Similar changes have been recorded with the muscle proteins [Weber, 1933; Weber & Stover, 1933]; in 45% urea myosin had an apparent molecular weight of 10^5 instead of about 10^6 and myogen one of 34,000 instead of 81,000. On the other hand, serum albumin, investigated in some detail by Burk [1932] and Pauli [1934], behaved differently. Burk discussed the corrections that must be applied because of departures from the idéal solution law and concluded that serum albumin had the same molecular weight in urea solution after denaturation as it had in water or in 75% glycerol. Burk [1937, 2; 1938] also showed that serum globulin and gliadin were denatured by urea without changing molecular weights. Huang & Wu [1930] denatured egg albumin in different ways and found differences between the osmotic pressures in urea solution, but Burk & Greenberg [1930] and Burk [1937, 1] found the same osmotic pressure for egg albumin whether it was dissolved in water or in 40% urea. The fact that Williams & Watson [1937] found a sedimentation constant for egg albumin in 50% urea corresponding to a molecular weight of 21,000 instead of the normal value of 36,000 at first sight contradicts Burk's results. However, all these results assume, that the particles of both native and denatured protein are spherical, and Bull [1940] has pointed out that the increase in viscosity of egg albumin on denaturation is evidence that the particles are no longer spherical. This conclusion is compatible with the observation of Lee & Wu [1932] that the area per molecule of films of native and urea-denatured egg albumin, making the usual assumptions about molecular weight and density, are 8400 and 10,400 sq. Å., but this increase in area was not confirmed by Bull [1938].

Too few observations have been made on the viscosities of native and urea-denatured proteins to permit any valid generalizations, but there appears to be an interesting difference between initially anisodimensional and viscous proteins and the more mobile proteins [cf. Hand, 1935]. Liu [1933], Neurath & Saum [1939] and Bull [1940] found a rise in the relative viscosity of the globular egg albumin on denaturation, whereas Frampton [1939] and Edsall & Mehl [1940] found that denaturation increased the mobility of the rod-shaped proteins

tobacco mosaic virus and myosin. Many workers have noticed the increased fluidity given by urea to glutinous products, and this property was used by Dold [1924] for liquefying sputum as a preliminary to examination for tubercle bacteria.

3. *Evidence on the nature and reversibility of the changes wrought by urea*

It is clear that the action of urea on a protein is complex and that the strength of the urea, the time of action and the temperature may determine which of the possible actions occurs. The milder changes, such as swelling, increase in solubility and permeability, and perhaps the attachment of urea to parts of the protein, are generally reversible [Heim, 1937]. Carpenter & Lovelace [1938], from a study of the rotatory dispersion, found with gelatin that the effects even of concentrated urea were completely reversible, but this is exceptional and with most proteins intense treatments cause a change that may properly be regarded as denaturation [cf. Mirsky & Pauling, 1936]. This change is again complex and proceeds through a succession of stages according to the severity of the treatment with urea. For example, Beck & Schormüller [1937] in studying the solution of horse meat in urea found that heating solutions of the already denatured protein had a further effect in preventing its precipitation on dialysis. Diebold and Juhling [1938] obtained similar results with fibrinogen, and Steinhardt [1938] found that only 30% of a sample of horse haemoglobin precipitated on dialysis after exposure to 4M urea whereas all precipitated after exposure to 7·46M. Edsall & Mehl [1940], extending the observation of von Muralt & Edsall [1930] on the loss of anisotropy of flow of myosin in strong urea solutions, commented on the fact that this loss occurred when the treatment was insufficient to reduce the viscosity to its minimum value.

Hopkins [1930] first showed that proteins varied in their resistance to denaturation by urea. The denatured protein is usually recognized by diluting or dialysing solutions until the urea is too dilute to keep it in solution. Steinhardt [1938] and Burk [1937, 2] have stressed the uncertainty of this criterion, partly because of the solubility of denatured protein in the presence of native protein and partly because of the readiness with which the first stages of denaturation may be reversed by dialysis. Steinhardt also presented evidence that denaturation of some proteins did not occur in the urea, even though dissociation had taken place, but that it was a result of subsequent treatments such as dilution or dialysis. On the other hand, Burk [1932] found that the heat coagulation of serum albumin could be partly reversed by dissolving it in urea and dialysing at a low temperature; Laporta [1932] made similar claims for other proteins.

For obvious reasons experiments on proteins with measurable specific activities would give the most satisfactory evidence that urea has had an effect that is irreversible by dilution or dialysis. As yet few of these have been made. The concentrations of urea used in experiments with pepsin and trypsin have not affected them irreversibly. The behaviour of fibrinogen is interesting, for Wöhlisch & Kiesgen [1936] found that in 30% urea it could no longer be coagulated by heat, alcohol or thrombin, whereas Meissner & Wöhlisch [1937] recovered from the solution fibrinogen that could be clotted. Diebold & Jühling [1938] examined the system in greater detail, finding that thrombin could act in the presence of 15% urea but that the precipitation of fibrin was prevented. A water-soluble protein that could no longer be clotted with thrombin could be made by more vigorous treatment with urea. The effects of urea on the activities of viruses and bacteria are described later in this paper.

Steinhardt's [1938] hypothesis that urea and other amides act by competing with peptide bonds in one part of a protein for association with neighbouring bonds in the protein grid is compatible with the evidence so far adduced. On this hypothesis the protein is held together by these associations and the probability that a protein will return to its original configuration on the removal of urea will depend on the number of these associations that have been broken and have to reform. With conjugated proteins, urea may split off the prosthetic group. Experience shows that this usually leads to greater instability of the protein moiety, so that denaturation of that might also be expected. Some agents, e.g. sodium dodecyl sulphate [Sreenivasaya & Pirie, 1938] may dissociate a prosthetic group without denaturing the protein, but no example is known of urea acting in this way, although it is presumably possible. On the other hand, examples of denaturation without the liberation of the prosthetic group are known.

The most characteristic chemical difference between native and denatured proteins is the presence in the latter of free —SH groups, or of groups that can readily be turned into —SH groups by reducing agents. This effect was first demonstrated by Hopkins [1930] with egg albumin and horse and sheep serum proteins after denaturation by urea. Burk [1937, 1] confirmed these results and showed that amandin, edestin, excelsin, sheep haemoglobin and myogen also gave —SH groups, but not gliadin, zein or pepsin. Quantitative measurements of the —SH groups formed by action of urea and related substances have been made by Greenstein [1938; 1939] on egg albumin and by Greenstein & Edsall [1940] on myosin. Not all the available —SH was liberated from myosin by prolonged treatment with urea, for on denaturation with guanidine twice the apparent cysteine content was found. Tobacco mosaic virus also gives an —SH reaction after denaturation with urea. Little other chemical work has been done on differences between native and urea-denatured proteins. Chou & Wu [1936] found that it had no effect on the formaldehyde titrations of five proteins, suggesting that the $-NH_2$ groups were not concerned in the changes, although Hopkins [1930] and Wu *et al.* [1931] noticed a change in *p*H on denaturation.

4. *The effects of urea on bacteria and tissues*

We have already referred to the first experiments on tissues in which urea was used as a solvent, and we conclude this survey by describing some systems in which it is probable, though not certain, that the changes accompanying exposure to urea result from its action on protein. Mustard and cress seeds exposed to 5% urea did not germinate [Ramsden, 1902]; *Esch. coli* barely grew in media containing 8% urea [Wilson, 1906] and was more easily lysed by its bacteriophage in the presence of 5% urea [Bronfenbrenner & Hetler, 1933]. Changes to filamentous forms were described by Wilson [1906] and by Péju & Rajat [1906] when some bacteria were grown on media containing 2–5% urea, but many bacteria, e.g. staphylococci, streptococci and sarcinae, were apparently unaffected. Symmers & Kirk [1915] measured the rate at which *Ps. pyocyanea* was killed by the presence of urea when suspended in blood, and found 25 g. per 100 ml. to be the lowest effective concentration. They recommend the liberal use of urea in the treatment of wounds and mention the use of solutions as mouth washes for diptheria carriers and for sterilizing tuberculous sputum. Individual bacteria differ widely in their resistance to concentrated urea solutions [Dold, 1924; Foulger & Foshay, 1935; Finger, 1937]; *Myco tuberculosis* was the most resistant tested and was killed by 3 hr. exposure to saturated urea at 37°.

Bacterial spores were much more resistant than bacteria. Rooschütz [1935] noted their presence in most commercial samples of urea, and Dold & Weyrauch [1924], having found spores to be viable after a month's exposure to saturated urea at room temperature, recommended the use of urea solutions in their isolation.

In popular medicine the use of urine and of plant extracts containing related substances such as allantoin, e.g. comfrey [Macalister, 1912], for promoting wound healing is some thousands of years old. Solid urea was used to prevent the suppuration of wounds by Symmers & Kirk [1915], but no further use of the treatment seems to have been made until Millar [1933] described its beneficial effects on sloughing cancers. Since then the successful use of solid urea on a large number of wound cases has been described by Holder & MacKay [1937; 1939], by Muldavin & Holtzmann [1938] and others. Strong solutions seem to be equally effective against chronic infections of the ear, nose and throat [Foulger & Foshay, 1935; Mertins, 1937]. Treatment with solid urea resembles in some ways the well-known treatment of wounds with fly maggots. Presumably part of the benefit in both treatments comes from the removal of damaged tissue, by solution with urea and by selective feeding with the maggots. It is unknown why urea does not dissolve healthy tissues, but this relatively specific action has been noted by Stoeltzner [1925] and Stoye [1925], who claimed that the injection of concentrated urea solutions under non-adherent scar tissue led to its ready removal and to the smooth healing of the wound. They found, however, that adhering scar tissue was apt to become necrotic with this treatment. Tissues are readily permeable to urea, and it may be that where there is an undamaged blood supply, a sufficiently high concentration of urea to dissolve tissue proteins cannot be maintained. In wound healing, bacteriostatic action is doubtless of great importance, but it has also been suggested, notably by Robinson [1938], that urea has a directly stimulating action on cell proliferation. Mond & Hoffman [1928] found that urea as dilute as 0·3 *M* damaged the surface of red blood corpuscles and tended to make lysis easy. Smadel *et al.* [1938] have also made similar observations, but this property does not seem to have caused any difficulties in the clinical use of urea.

5. *The effect of urea on viruses*

Burnet [1933] tested the resistance of 24 different dysentery coli bacteriophages to urea and divided them into three groups according to whether they were rapidly inactivated, slowly inactivated or unaffected by 27·7% urea; he suggested that the smaller phages were the more resistant. McKay & Schroeder [1936] found that rabies and anterior poliomyelitis viruses lost both their ability to infect and to immunize rabbits after exposure to 40% urea. Hoyt & Warner [1940] confirmed the inactivation of rabies virus, but found that after less severe treatment, so that it was not completely inactivated, it retained its immunizing power. Smadel *et al.* [1938] found that in 22% urea there was an initial rise in the sedimentation constant of vaccine virus followed by a fall, whereas in 40% the fall was continuous. Inactivation was complete in 3 days in 22% urea, whereas a 10–15% solution was without any apparent effect. These changes presumably correspond to the swelling that has been observed with some other proteins in dilute urea and with the denaturation that occurs in concentrated solutions. Höring [1939] showed that concentrated urea quickly inactivated yellow fever virus, whereas in dilute urea the virus was about as stable as it was in water.

Bawden & Pirie [1937] found that three strains of tobacco mosaic virus were more resistant to urea than were the proteins used by Hopkins [1930] or than some of the phages used by Burnet [1933]. No details were given, though the denaturation of the viruses and the loss of liquid crystallinity after several hours' exposure to saturated urea were mentioned. Mehl [1938] also mentioned the loss of anisotropy of flow of tobacco mosaic virus preparations after treatment with urea. Frampton & Saum [1939] and Frampton [1939] reported that solution of tobacco mosaic virus in 6 *M* urea and 0·1 *M* phosphate buffer caused a hundredfold increase in the diffusion constant with no change in infectivity. They interpreted this result as indicating that the urea caused the virus particles to disaggregate into single molecules of molecular weight around 100,000. They suggested that these were the true virus molecules and that the large particles with weights equivalent to molecular weights of many millions were merely aggregates. However, no increase in infectivity accompanied the urea treatment such as would be expected from such a disaggregation into small infective units, and later work has not confirmed this view. Stanley & Lauffer [1939] were unable to obtain any evidence that the low molecular weight protein produced by the action of urea was infective. They found that the disintegration depended on the salt content, *p*H and temperature, and any residual activity in treated virus preparations was always found to be associated with remaining protein of high molecular weight. Measurements in the ultracentrifuge [Martin, 1939] indicated that the inactivated virus had a molecular weight of 400,000, but osmotic pressure measurements [Stanley & Lauffer, 1939] showed that on continued action of urea it fell to 40,000.

Experimental

1. *Materials and methods*

In this paper experiments are described with the four viruses, tobacco mosaic, tomato bushy stunt, potato "X" and tobacco necrosis. The virus preparations used were made by the methods described previously [Bawden & Pirie, 1937; 1938, 1; 1938, 2; Pirie *et al.* 1938]. The tobacco necrosis virus used probably differs from that used earlier, for although the preparations used were made by the methods described then we have been unable to get any crystalline material. Instead, the whole preparations have consisted of amorphous material closely resembling the amorphous two-thirds of the previous preparations. The serological reactions of our preparations also differ from those of the earlier preparations. It seems probable that the virus culture used in 1938 was a mixture of strains, of which only one was crystallizable, and that in repeated transfers during the last two years this strain has been lost.

Because of shortage of material only a few tests were made with potato virus "X" and tobacco necrosis virus. Most were made with tobacco mosaic virus, because it is both readily obtained in large quantities and shows the convenient property of anisotropy of flow. Qualitative observations on the loss or diminution of this property, made by shaking solutions in test tubes, 0·5–1 cm. in diameter, between crossed polarizers, give valuable preliminary indications of the severity of treatment necessary to distintegrate the virus. This provides quite a sensitive test, for the disintegration products not only themselves fail to show anisotropy of flow but also impede the orientation of the still undamaged virus so that it will no longer form a liquid crystalline layer and so that a greater rate of shear is necessary to get maximum orientation. Some treatments destroy the infectivity of tobacco mosaic virus without disintegrating

it. These treatments also leave the anisotropy of flow and serological reactions unimpaired, but urea has not been found to do this.

For simplicity the purified materials used in our tests will be called viruses, although their exact relationships with the viruses as they occur in infective saps is still uncertain. With tobacco mosaic virus and potato virus "X" it is known that purification by the methods used leads to a reduction in infectivity and to some other changes, which have been attributed to the linear aggregation of virus particles [Bawden & Pirie, 1937; 1938, 1, 2, 3]. Except for tobacco necrosis virus, which was tested only for changes in infectivity, the treated virus preparations were tested both for their infectivity and serological activity. In the infectivity tests dilutions were made in 0·1 *M* phosphate buffer at *p*H 7, and local lesion counts were made at two dilutions of the inocula, usually at 10^{-4} and 10^{-5} g. protein per ml. The host used for lesion counts for tobacco mosaic and tomato bushy stunt viruses was *Nicotiana glutinosa*, for potato virus "X" *N. tabacum*, var. White Burley, and for tobacco necrosis virus *Phaseolus vulgaris*, var. Canadian Wonder. To reduce to a minimum the errors arising from differences in the susceptibility of individual plants, infectivity tests were all arranged in the form of Latin squares or incomplete blocks [Youden, 1937]. In each test at least six leaves were inoculated with each dilution of the preparation tested. As solutions containing the same quantities of active virus can often give widely different numbers of local lesions if they contain different amounts of salts or other materials, the compositions of all test and control inocula were adjusted to be at the same *p*H, and to contain the same amounts of urea, salts and protein before inoculation.

The dilutions in the serological tests were made in 0·85% NaCl solution. 1 ml. of antiserum at a dilution of 1/50 was added to a series of tubes each containing 1 ml. of virus solution at different concentrations, and the smallest amount of virus in g. to give a precipitate visible to the eye in 2 ml. of such mixtures is recorded as the serological titre. The serological titres of different preparations of tomato bushy stunt virus are remarkably constant, but different preparations of tobacco mosaic virus and of potato virus "X" give widely different titres. The titre of any one preparation remains constant and reproducible, changes in it accurately reflecting changes in the virus. Therefore, as the tests described were carried out over a long period and with different virus preparations, although the serological titres given in any one table are strictly comparable, they cannot necessarily be compared with those in the other tables or with those previously published.

2. *Effect of different concentrations of urea*

It is well known that the rate at which a disinfectant works is a non-linear function of its concentration [Smith, 1921], and that below a critical concentration it has no appreciable effect. This is also true for the killing of bacteria by urea, for which Wilson [1906] and Symmers & Kirk [1915] have found the critical range of concentration to be from 8 to 25%, i.e. from 1·33 to 4·17 *M*. Tables 1–4 show the effects of variations in the urea concentration on the rate of inactivation of the four viruses. They show clearly that concentrated urea has had an effect on the specific activities of all the viruses that is not reversed by dilution. They also show that the individual viruses differ widely in their resistance to the inactivating action of urea, and that, as with bacteria, there is for each one a critical concentration below which there is little or no inactivation. With all the viruses, the reduction in infectivity is approximately proportional to the reduction in serological activity, and with none is there any indication

that the proteins with low molecular weights produced by the action of urea on the large virus particles have any specific virus activities as suggested by Frampton & Saum [1939] for tobacco mosaic virus. In establishing the lack of action, or more probably the reversibility of the action, of dilute urea on the viruses the results also show that it is legitimate to study the kinetics of the action by diluting the mixtures largely when the action is to be stopped.

Table 1. *Effect of concentration of urea on inactivation of tobacco mosaic virus*

Urea *M*	ml. of phosphate and urea mixed to get molarity: Phosphate	ml. of phosphate and urea mixed to get molarity: Urea	ml. of phosphate and urea added in diluting: Phosphate	ml. of phosphate and urea added in diluting: Urea	Serological titre	Average no. of lesions at: 10^{-4}	Average no. of lesions at: 10^{-5}
8	0·0	0·8	2·0	0·0	1/5000	1	0
7	0·1	0·7	1·9	0·1	1/10,000	2	0·5
6	0·2	0·6	1·8	0·2	1/40,000	15	3
5	0·3	0·5	1·7	0·3	1/80,000	24	5
4	0·4	0·4	1·6	0·4	1/320,000	65	28
3	0·5	0·3	1·5	0·5	1/640,000	116	44
	Control; urea diluted before adding virus				1/640,000	117	54

To 0·2 ml. of 1·64% tobacco mosaic virus was added sufficient *M*/10 phosphate at *p*H 7·1 and 10 *M* urea solution to give 1 ml. of solution at the required molarity. After 20 hr. at 15° each was diluted with phosphate and urea to a final volume of 3 ml., i.e. to 2·67 *M* urea, and tests were made 36 hr. later.

Table 2. *Effect of concentration of urea on inactivation of tomato bushy stunt virus*

Urea *M*	Serological titre	Average no. of lesions per leaf at: 10^{-4}	Average no. of lesions per leaf at: 10^{-5}
8	No ppt. at 1/5000	0	0
7	1/10,000	12	6
6	1/160,000	108	47
5	1/640,000	137	82
Control	1/640,000	157	79

The samples were prepared as in Table 1 and were exposed for 20 hr. at *p*H 8 and 15°. They were then diluted till the urea was 1·6 *M* and tested 36 hr. later.

Table 3. *Effect of concentration of urea on inactivation of potato virus "X"*

Urea *M*	Time	Serological titre	Average no. of lesions per leaf at: 10^{-4}	Average no. of lesions per leaf at: 10^{-5}
7	10 min.*	No ppt. at 1/10,000	0	0
6	1 hr.*	No ppt. at 1/10,000	1	0
4·5	1 hr.*	No ppt. at 1/10,000	6	1
3	1 hr.*	1/40,000	63	15
1·2	36 hr.	1/320,000	133	110
	Control	1/640,000	220	190

* These preparations spent an additional 36 hr. exposed to 1·2 *M* urea.

In all tests 0·2 ml. lots of 0·48% salt-free potato virus "X" were diluted with the volumes of water and 10 *M* urea in 0·1 *M* phosphate buffer at *p*H 7·1 required to give 0·5 ml. at the required molarity of urea. After the stated time at 17° they were diluted to 1·2 *M* urea and tested 36 hr. later.

Table 4. *Effect of concentration of urea on the inactivation of tobacco necrosis virus*

Urea *M*	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
7·5	0	0
5·5	10	2
3·5	82	20
Control	240	130

The samples were exposed for 20 hr. at *p*H 7 and 16°.

3. *Effect of temperature on the rate of inactivation*

The fact that proteins have a large temperature coefficient of denaturation makes Hopkins's [1930] discovery that the denaturation of egg albumin in the presence of urea proceeds faster at 0° than at 37° exceptionally interesting. Stanley & Lauffer [1939] found that the disintegration of tobacco mosaic virus in urea proceeded more rapidly at 0° than at 25°, but that it proceeded more rapidly at 40° than at 25°. We have confirmed this result with tobacco mosaic virus, and have found that potato virus "X" and tomato bushy stunt and tobacco necrosis viruses also have temperature ranges in which cooling leads to an increase in the rate of denaturation by urea. Table 5 shows the results of an

Table 5. *Effect of temperature on the denaturation of tobacco mosaic virus by urea*

Temperature	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
0°	1/100,000	13	4
9·5	1/400,000	20	6
20	1/800,000	31	7
37	1/200,000	6	2
55	No ppt. at 1/50,000	0	0
Control	1/1,600,000	40	12

Each sample, except the control, was exposed to 6 *M* urea for 4 hr. in the presence of *M*/30 phosphate buffer at *p*H 7·0 and the temperature stated, they were then diluted for testing. The control was diluted immediately after mixing at 15°.

experiment on the denaturation of tobacco mosaic virus by urea between 0° and 55°, and Table 6 those for a more detailed experiment between −10° and +11°. Results of experiments at different temperatures with potato virus "X" and tomato bushy stunt virus are given in Tables 7 and 8.

The viruses used in these temperature tests vary widely in their reactions to freezing and drying, presumably because they hold water in different ways. Bushy stunt virus in salt-free solution is denatured and inactivated by freezing and thawing, whereas tobacco mosaic virus and potato virus "X" are not, and bushy stunt virus and potato virus "X" are completely inactivated when neutral solutions are dried, whereas tobacco mosaic virus is only partially inactivated. In spite of these differences in the properties of the individual viruses, the denaturation of all of them by the urea shows a similar increase in rate at low temperatures. The remote possibility exists that exposure of virus solutions to low temperatures alone might have some effect; for example, exposure to −10° might inactivate tobacco mosaic virus even though freezing at

Table 6. *Denaturation of tobacco mosaic virus by urea between −10° and 11°*

Urea *M*	Time of exposure hr.	Temperature	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
6	24	11°	1/50,000	3·1	0·8
		0	1/20,000	2	0·2
		− 5	1/10,000	0·5	0·1
		−10	No ppt. at 1/5000	0	0
	48	11	1/40,000	5·2	0·6
		0	1/10,000	0·7	0
		− 5	1/5000	0·1	0
		−10	No ppt. at 1/5000	0	0
5	24	11	1/200,000	30·7	6·6
		0	1/100,000	18·4	2·5
		− 5	1/20,000	3·6	0·4
		−10	1/8000	0·8	0·2
	48	11	1/100,000	14·1	0·5
		0	1/80,000	8·5	1·4
		− 5	1/20,000	6·0	0·5
		−10	1/5000	1·7	0·1
Control; urea diluted before adding virus			1/800,000	67·1	32·3

0·2 ml. samples of a 1·64% solution of tobacco mosaic virus were added to 0·8 ml. of 7·5 or 6·25 *M* solutions of urea in *M*/30 phosphate buffer at *p*H 7 and already at the temperature stated. After the stated time at the given temperatures, those exposed to 6 *M* urea were diluted with 2 ml. of water and those exposed to 5 *M* with 1·9 ml. of water and 0·12 ml. of 10 *M* urea. After 36 hr. at room temperature they were diluted further for testing.

Table 7. *Effect of temperature on the inactivation of potato virus "X" by urea*

Urea *M*	Temperature	Serological titre	Average no. of lesions per leaf at 10^{-4}
3	37°	1/40,000	15
	10	1/80,000	25
	−10	1/20,000	10
2	37	1/160,000	43
	10	1/320,000	101
	−10	1/30,000	12
	Control; urea diluted before adding virus	1/640,000	176

Samples of 0·24% solution of potato virus "X" exposed in *M*/30 *p*H 7·0 phosphate buffer at the temperatures stated for 1 hr. to the stated concentration of urea. Samples were then diluted to 0·5 *M* urea and left for 36 hr. at room temperature before testing.

Table 8. *Effect of temperature on inactivation of tomato bushy stunt virus by urea*

Temperature	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
37°	1/160,000	71	30
20	1/320,000	135	58
0	1/80,000	35	11
−12	1/20,000	0	0
Control; urea diluted before adding virus	1/320,000	145	80

Samples were exposed for 7 hr. in 6 *M* urea at *p*H 7·9 at the stated temperatures. They were then diluted to 2 *M* urea and left for 36 hr. at room temperature before testing.

this temperature does not. To test this possibility eight samples of 0·47% tobacco mosaic virus solution in *p*H 6·9 phosphate:borate buffer mixture [Kolthoff, 1925] were mixed with 2 vol. of either 6*M* urea, 6*M* acetamide, 6*M* formamide or 6*M* alcohol. One of each pair of these mixtures was kept at $-10°$ and the other at 16°. All were then diluted until the urea or other substance was 1·5*M*, and they were tested 36 hr. later. The serological titres of the two samples containing urea were 1/10,000 for that kept at $-10°$ and 1/640,000 for that kept at 16°, whereas all the other samples gave a titre of 1/2,560,000. Similarly, the effect of freezing solutions of tomato bushy stunt virus is a direct effect of the freezing and not one of the low temperature, for when infected leaves, infective sap or solutions of purified virus containing salts, are cooled below 0° the virus is not inactivated. The increased rate of denaturation at low temperatures seems to be an effect specific to urea, for other denaturants have been tested over a range in temperature and none has been found that inactivates more rapidly at $-10°$ than at higher temperatures.

4. *Effect of* p*H changes on the rate of inactivation*

Hopkins [1930] showed that egg albumin was denatured in the *p*H range 5–6·5, but he made no detailed tests on the rate of denaturation by urea at different hydrogen ion concentrations. Stanley & Lauffer [1939] found that there was little or no denaturation of tobacco mosaic virus by 6*M* urea at *p*H 5·5, that it was more rapid at *p*H 8·2 than at *p*H 7·4 and that it was much slower at *p*H 6·4 than at *p*H 7·4. We have confirmed the effect of alkali in increasing the rate of inactivation of tobacco mosaic virus by urea, and have found tomato bushy stunt virus to behave similarly.

The *p*H of a buffer solution in the presence of concentrated urea is a matter of some uncertainty. Burk & Greenberg [1930] found that in 6·66*M* urea the apparent *p*K of acetic acid shifted from 4·6 to 5·2 and that of phosphoric acid from 6·8 to 7·2. Hopkins [1930] noticed an increase of 0·8 to 1·0 units in the *p*H of protein solutions on the addition of urea. There are probably at least three causes of this effect: (1) the *p*K shift noticed by Burk & Greenberg, (2) the *p*H shift of protein solutions when denatured [Wu *et al.* 1931] and (3) the presence of ammonium salts in most samples of urea and the gradual production of more of these on standing, especially if the solution is heated [Lewis & Burrows, 1912; Ogiu & Pauli, 1932; Beck & Schormüller, 1937]. In our experiments two methods were adopted for measuring the *p*H. In the first, 10*M* urea in *M*/10 phosphate buffer was added in suitable quantities to the virus solutions and the *p*H recorded by the hydrogen electrode after the mixture was diluted 10-fold (the apparent *p*H before such dilution was about 0·2 units higher). In the second, a virus : buffer mixture of the required *p*H value was mixed with a neutral solution of urea.

Table 9 shows the results of one experiment on the effect of *p*H on the inactivation of tobacco mosaic virus by urea. The critical range of *p*H within which the rate of inactivation of tomato bushy stunt virus increases rapidly is even narrower than that with tobacco mosaic virus, and is shown in Table 10. In this table, and in Table 8, it appears at first sight that urea treatment is destroying infectivity without proportionally affecting the serological activity. However, we have already shown [Bawden & Pirie, 1938, 2] that this is one of the effects of treating bushy stunt virus with alkali alone, although then it occurs only at a higher *p*H. Thus it seems that in the presence of urea this type of inactivation can occur in less alkaline solutions, but the effect is partially masked by secondary reactions that destroy both infectivity and serological activity.

Table 9. *Effect of changes in pH on the inactivation of tobacco mosaic virus in the presence of urea*

Urea *M*	*p*H	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
6	5·16	1/2,560,000	220	98
6	6·07	1/1,280,000	160	48
6	6·76	1/160,000	50	8
6	7·87	No ppt. at 1/1000	0	0
6	9·16	No ppt. at 1/1000	0	0
4	9·16	1/4000	3	1
0	9·16	1/64,000	180	80
Control; urea diluted before mixing		1/2,560,000	260	140

The samples were exposed for 20 hr. at 15°. 0·1 ml. of a 3·3% solution of tobacco mosaic virus solution was mixed with 0·3 ml. of a mixture of *M*/10 phosphate and *M*/20 borate made up according to Kolthoff [1925] to the *p*H stated. 0·6 ml. of 10 *M* urea was added to the top 5 samples, 0·4 ml. plus 0·2 ml. of water to sample 6 and 0·6 ml. of water to the last sample. After 20 hr. the requisite amount of borate or phosphate was added to each sample to bring the *p*H to 6·76, and water and urea were added to the last 2 samples to bring the volume to 3·35 ml. and urea concentration to 1·79 *M*. Tests were made 36 hr. later.

Table 10. *Effect of pH on the inactivation of tomato bushy stunt virus in the presence of 7·7 M urea*

*p*H	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
7·0	1/320,000	71	16
8·1	1/40,000	0	0
9·16	No ppt. at 1/10,000	0	0
Control; urea diluted before adding virus	1/320,000	55	15

0·15 ml. lots of Kolthoff buffer at the *p*H stated and containing 1 mg. of bushy stunt virus were mixed with 0·5 ml. of neutral 10 *M* urea. After 80 min. at 18·5° 2 ml. of water and 0·15 ml. of *M* acetate buffer at *p*H 4 were added, and tests were made after 36 hr.

5. *Comparison of purified virus with clarified infective sap*

The experiments already described were made with purified viruses prepared by precipitation methods. It is known with potato virus "X" and tobacco mosaic virus that these methods cause a fall in infectivity and filterability, and we have suggested [Bawden & Pirie, 1937] that this arises from an aggregation of the virus into particles of a greater length/width ratio. The most plausible explanation for this is that the virus particles as they occur in the plants have materials at their ends which keep them from aggregating, and that during the purification processes these end materials are removed. In view of the known differences between tobacco mosaic virus in infective sap and in the purified state, Martin's [1939] statement that the former is more readily inactivated by urea is of some interest. Martin has published no details of his experiments and we have therefore been unable to duplicate them, but in the tests we have made comparing the behaviours of crude and purified preparations the increased stability of the latter is only slight.

Leaves from infected tobacco plants were frozen and minced, and the sap expressed through muslin and centrifuged. The *p*H of the supernatant fluid,

measured by the hydrogen electrode, was 5·9. A 4% solution of $Na_2HPO_4,12H_2O$ was made with this sap, which, after centrifuging off the precipitate formed, had *p*H 6·9. As infective sap usually contains 1–2 g. of virus per litre, a comparison solution was made containing 1·57 g. of purified virus per litre in *M*/10 phosphate buffer at *p*H 6·9. Both solutions were then made up to 6*M* urea by the addition of solid urea, and after the required times at 15° samples were taken and diluted to 2*M* urea for testing later. To obtain 100 ml. of 6*M* urea, 73·5 ml. of solution were added to 36 g. of urea, so that the final concentration of purified virus in the mixtures was $1{\cdot}57 \times 0{\cdot}735$, or 1·15 g. per litre. To the control solutions only sufficient urea was added to give a concentration of 2*M*. Table 11 gives the

Table 11. *Comparison of the rates of inactivation of purified and crude tobacco mosaic virus preparations at* p*H 6·9 in 6*M *urea*

	Phosphate: infective sap mixture			0·115% solution of purified virus in phosphate buffer		
		Lesions			Lesions	
Time	Serological titre	10^{-4}	10^{-5}	Serological titre	10^{-4}	10^{-5}
30 min.	1/81	43	14	1/243	144	64
2·5 hr.	1/27	6	1·5	1/27	36	14
20 hr.	0	0	0	1/3	3	0
Control	1/729	215	158	1/2187	204	132

Description in text.

results of this comparison. It again illustrates the greater infectivity for a given serological titre that is characteristic of unpurified virus. It also shows that the inactivation of the virus in infective sap proceeds somewhat faster and farther than that of the purified virus, but the difference is slight and may well be caused by differences in the salt contents of the two preparations and by the action of the urea on the salts. Alternatively, it is possible that at the same time as the urea is denaturing the purified virus it also dissociates the aggregates formed during the purification processes, and that this slight increase in the number of infective units partly masks the inactivating effect and accounts for the slower inactivation of the purified virus.

6. *Chemical and physical changes accompanying inactivation by urea*

No detailed study has been made of the changes taking place during the inactivation of the viruses by urea, but the two anisotropic viruses with low nucleic acid content behave differently from the two isotropic viruses with higher nucleic acid contents. Stanley & Lauffer [1939] found that the inactivation of tobacco mosaic virus by urea led to a separation of the nucleic acid from the protein moiety. We have confirmed this, and find that nucleic acid is also liberated from potato virus "X" during the inactivation. On the other hand, the precipitates that separate from preparations of tomato bushy stunt and tobacco necrosis viruses denatured by urea still contain nucleic acid.

The solvent action of urea on native and denatured proteins was described in the introduction. The interrelation of these two actions with fibrinogen was studied by Diebold & Jühling [1938], who found that after a few hours at 37° solutions in 0·9% NaCl had a minimum opacity when they contained 12% of urea and a maximum when they contained 20%. Below 12% the lyotropic effect of urea on native fibrinogen was being measured and above 20% its solvent action on denatured fibrinogen, while between these two concentrations

denaturation and partial precipitation were proceeding. In their behaviour towards urea bushy stunt and tobacco necrosis viruses to some extent resemble fibrinogen. Tomato bushy stunt virus is almost completely inactivated by exposure to 7 *M* urea for 20 hr. at *p*H 8 and room temperature, but at this urea concentration, in the presence of a trace of salt, the denatured protein is insoluble, and it dissolves only slowly in 8–9 *M* urea. On the addition of a trace of salt to solutions exposed for 10–20 hr. to 6–8 *M* urea there is an immediate, apparently irreversible precipitation. These precipitates, like the original virus, contain 5% of carbohydrate as estimated by heating with orcin and sulphuric acid. The carbohydrate can be removed from the denatured protein by extraction with boiling dilute ammonium hydroxide, as it can from virus denatured by heating or drying [Bawden & Pirie, 1938, 2]. Tobacco necrosis virus behaves similarly but the effect is more easily demonstrated as inactivation occurs with more dilute urea. The precipitate of denatured virus is also more readily soluble in urea, so that there is an immediate disappearance of the faint opalescence characteristic of the virus preparation when enough urea is added to a 0·12% virus solution to make it 8 *M*. In more dilute urea solutions, from 4 to 6·5 *M*, there is rapid precipitation, but after some hours the precipitated material dissolves. On diluting any of these solutions of inactivated tobacco necrosis virus with 0·9% NaCl solution a precipitate separates that still contains nucleic acid.

The absorption spectra of the more highly purified virus preparations have all shown a characteristic maximum at about 260 mμ, i.e. in the region associated with absorption by nucleic acid. As part of a general examination of the manner of linkage of the nucleic acid to the protein moiety we have compared the absorption spectra of the separated protein and nucleic acid with that of the intact virus. These experiments have shown that processes leading to separation of the nucleic acid lead to diminution of the absorption in the ultra-violet. This result is not unexpected, for it is well known that the intensities, and even the position of, the absorption maxima of purines and pyrimidines are affected by substitution or by changes in ionization [cf. Ellinger, 1938]. Furthermore, the treatments effecting the separation increase the clarity of the fluids, presumably because the average molecular weights of the dissolved materials are less, and this reduces the amount of light scattered, especially in the ultra-violet range. It is reasonable to suppose that much of the apparent absorption by plant virus preparations in the ultra-violet is caused by scattering. Lavin *et al.* [1939] found that tobacco mosaic virus preparations made by precipitation with ammonium sulphate absorbed more strongly than those made by ultra-centrifuging, and, as the former are the more highly aggregated, part at least of this effect may be caused by the difference in scattering.

Urea dissociation of potato virus "X" illustrates the effect on the absorption spectrum of the separation of the nucleic acid from the protein and the breakdown of the protein into smaller particles. When we described the purification of virus "X" [Bawden & Pirie, 1938, 1] we did not publish a spectrum, but in a later paper [Bawden & Pirie, 1939] preparations were described that had no absorption minimum at 245 mμ. By repeated differential high speed centrifuging of chemically purified products we now get preparations with a minimum at 245 mμ, although it is not so pronounced as those found with tobacco mosaic, tomato bushy stunt or tobacco necrosis viruses. This agrees with the figures published by Lavin *et al.* [1939]. The curves in Fig. 1 give the density of a 2 cm. layer of a 0·029% solution of potato virus "X" in the presence of 0·77 *M* urea and 0·08 *M* phosphate buffer at *p*H 7. For the upper curve, *A*, the virus was added to urea at 0·77 *M* and the spectrum taken immediately. For the lower

curve, *B*, the solution was made by adding the virus to a concentrated urea : phosphate mixture so that it was exposed to 7·1 *M* urea. After 40 min., sufficient time for complete inactivation, the mixture was diluted to 0·77 *M*. Short exposure to 0·77 *M* urea apparently had no effect on the virus, for the curve *A* was identical with that given by the virus dissolved in water.

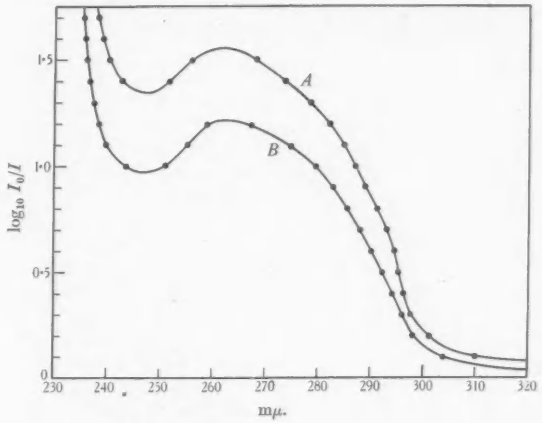

Fig. 1.

Changes similar to those with potato virus "X" occur when tobacco mosaic virus is inactivated with urea. On the other hand, absorption in the ultra-violet by tomato bushy stunt preparations is increased by denaturation with urea. The protein and nucleic acid are not separated when bushy stunt virus is treated with urea and the inactivated virus is insoluble in urea. Either of these differences between bushy stunt and the other two viruses may explain the difference in the behaviour of its absorption spectrum, for although in the absence of salts there is no actual precipitation of denatured protein, the fluid becomes more opalescent. When bushy stunt virus is inactivated by sodium dodecyl sulphate the protein and nucleic acid are separated and remain water-soluble, and there is an increase in the clarity of the solution. After this treatment the intensity of absorption of ultra-violet is increased, so that from this point of view the inactivation of bushy stunt by sodium dodecyl sulphate resembles the inactivation of tobacco mosaic virus and of potato virus "X" by urea.

Discussion

In the experiments described in this paper urea has been regarded as the chief agent in causing denaturation. However, as denaturation requires the simultaneous control of several variables, this distinction is arbitrary. For example, as the rate of denaturation closely depends on the *p*H, it is as logical to regard urea as increasing the rate of alkaline inactivation as it is to regard alkali as increasing the rate of urea denaturation. Actually there is probably considerable interaction between the various factors. For example, our results show that at some *p*H values in the alkaline range, which do not themselves inactivate tobacco mosaic virus, concentrations of urea, which in neutral

solution do not inactivate, cause rapid inactivation. Also, one of the features of alkaline inactivation of tomato bushy stunt virus, loss of infectivity without loss of serological activity, occurs in less alkaline solutions in the presence of urea. Similarly, Drabkin [1939] compared the rates of denaturation of haemoglobin in dilute alkali, in 6*M* urea, and in a mixture of the two, and found that the mixture acted 60 times as rapidly as either component alone.

The restriction of the work reported to inactivation in the presence of urea is also largely arbitrary, for it is reasonable to assume that urethane, guanidine and related substances act in a similar manner and that there is a smooth transition to the somewhat remote denaturing agents such as pyridine, benzoates, salicylates, phenol and soaps. The effects of these substances on the plant viruses will be described in a later paper, but as little or nothing is known of the mechanism of their action it would be premature to attempt to classify them. There is one feature of inactivation in the presence of urea, however, that might justify the separation of urea from other denaturing agents and justify the view that urea is the chief agent in causing the denaturation rather than the other factors such as *p*H. This is the large increase in the rate of denaturation produced by cooling at temperatures below about 20°. This was found to be true for all the viruses we have tested, as well as for other proteins, but no such effect has been found for inactivation by alkali or other denaturing agents.

In our experiments only changes irreversible on dilution or dialysis and resulting in loss of specific virus activities have been investigated. The results show that for each virus there is a critical concentration of urea below which irreversible changes do not occur. This concentration varies with the different viruses, which, although they are all nucleoproteins, also differ in the manner in which they break down on denaturation. For example, the inactivation of potato virus "X" and tobacco mosaic virus is accompanied by the separation of the nucleic acid from the protein and the products are soluble in urea solution, whereas the inactivated bushy stunt and tobacco mosaic viruses are insoluble in urea and the precipitates still contain nucleic acid. In addition to these irreversible changes it is probable that urea can cause changes that are readily reversed and have no effect on virus activity. Frampton [1939] found that the addition of as little as 1*M* urea to solutions of tobacco mosaic virus greatly reduced the viscosity. As inactivation and disruption of the virus particles occur only slowly in much more concentrated urea solutions, it is probable that the immediate effect on viscosity is caused by the hydration of the particles and not by denaturation. We have found no evidence that the small particles produced by the disruption of the viruses possess virus activity, although the fact that purified virus is inactivated rather more slowly than impure virus may be evidence that the urea can dissociate the aggregates formed during purification as well as disrupt the actual virus particles.

Summary

The literature on the effects of urea on proteins, tissues, bacteria and viruses is reviewed. The four viruses, tobacco mosaic, potato "X", tomato bushy stunt and tobacco necrosis, are irreversibly denatured by urea. The denaturation is accompanied by loss of infectivity and serological activity. For each virus there is a critical concentration of urea below which there is no irreversible effect on infectivity. This concentration is smallest for potato virus "X" and greatest for tomato bushy stunt virus. The rate of inactivation is greatly increased by the presence of alkali. The rate of inactivation is minimum at about 20° and is much increased by cooling to −10°. The inactivation of purified tobacco mosaic

virus by urea proceeds only slightly more slowly than that of virus in crude infective sap. The inactivation of tobacco mosaic virus and potato virus "X" is accompanied by separation of the nucleic acid and protein, but the inactivation of bushy stunt and tobacco necrosis viruses is not. Changes in the absorption spectra that accompany inactivation are described.

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151. THE EFFECTS OF ALKALI AND SOME SIMPLE ORGANIC SUBSTANCES ON THREE PLANT VIRUSES

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(*Received 30 July 1940*)

Introduction

At the end of last century it was widely recognized that many substances, notably choline [Mauthner, 1874], sodium myronate and the mustard oils, could dissolve otherwise insoluble proteins, and this property was applied clinically by some workers. Hebra [1892] and Mendel [1905] recommended thiosinamine (sold dissolved in sodium salicylate under the name "Fibrolysin") for the softening and ultimate removal of scar- and fibrotic-tissue, and Fraenkel [1915] also recommended choline for this purpose. The clinical use of urea, to which we have previously referred [Bawden & Pirie, 1940], seems to have been developed independently of these observations. Spiro [1900] quoted many of the scattered observations of the early workers and described the solvent action, demonstrated either by dissolving a coagulum or by raising the temperature of heat coagulation, of urea, choline, piperidine, pyridine, urethane, formamide and mustard oils. He concluded that some of these acted simply as bases but that others formed compounds with the proteins. He [1900; 1904] also observed that some of these agents were protein precipitants when dilute although they were solvents when concentrated. A similar effect was noticed by von Furth [1896] during a study of the coagulation of muscle and serum proteins by salicylate and several alkaloids and it has been observed and commented on by many workers since. Neuberg [1916] studied the effects of salts of 43 organic acids, mainly aromatic, on the solubilities of casein and several other substances normally difficult to dissolve, and he found that benzoates, hippurates and many substituted benzoates increased solubility greatly. Recent papers have tended to be less extensive, concentrating on the action of one substance or on a group of closely related ones. Stoeltzner [1925], Hopkins [1930], Pauli & Weiss [1931], and Edsall & Mehl [1940], however, have compared the actions of a wide range of substances on a small number of test objects.

Svedberg and his colleagues studied protein solutions containing protamines, arginine or lysine, in the ultracentrifuge, and found that the proteins were often dissociated into apparently homogeneous particles of smaller molecular weights. This was a valuable confirmation and extension of the osmotic pressure measurements made on proteins dissolved in urea and acetamide. Details of the experiments have not been published [cf. Svedberg & Pedersen, 1940], but from the published summaries [Svedberg, 1937; Lundgren, 1938; Pedersen, 1938] it is clear that there was considerable specificity of action. For example, in the

presence of ammonium chloride arginine dissociated serum albumin but not *Helix* haemocyanin, whereas lysine dissociated the haemocyanin but not the albumin, and neither acted without the ammonium chloride.

In the experiments described in this paper purified preparations of the three plant viruses, tobacco mosaic, tomato bushy stunt and potato "X", were used. The methods of preparation and of testing were the same as in the experiments with urea [Bawden & Pirie, 1940]. For a number of reasons the results obtained with these purified virus preparations may differ from those that might have been obtained with crude preparations. First, the viruses may have undergone physical or chemical changes during purification. Secondly, the large amounts of normal plant proteins and other materials in infective saps might remove the agents added and so protect the viruses from their effects. Thirdly, concentrations of the agents too dilute to affect the viruses directly may produce a coagulum of normal plant protein and still active virus may be adsorbed on to and removed by this.

Experimental

Sodium dodecyl sulphate and alkali

The hydrogen sulphate of dodecyl alcohol (S.D.S.) was used by Bawden & Pirie [1938, 1, 2] to inactivate potato virus "X" and tomato bushy stunt virus and it was found to separate the nucleic acid from the proteins. The work was extended to tobacco mosaic virus by Sreenivasaya & Pirie [1938], who made a more detailed study of the nucleic acid and the water-soluble, phosphorus-free protein produced by its action on this virus. Of the three viruses, potato virus "X" is the most susceptible to the action of S.D.S. and tomato bushy stunt virus the least. Even potato virus "X", however, is more resistant than other proteins that have been tested. S.D.S. has little effect on this virus at dilutions greater than 0·3 % in neutral solution, although changes in haemoglobin [Anson, 1939], phyllochlorin [Smith, 1940] and cytochrome *c* [Keilin & Hartree, 1940] have been brought about rapidly at 10 times this dilution. Analogous changes are caused by some other surface active substances, e.g. soaps and bile salts, and these have been discussed in the papers quoted.

Sreenivasaya & Pirie [1938] found that tobacco mosaic virus was almost inactive after 24 hr. in 1 % S.D.S. at 37° and *p*H 8. If either the temperature or the *p*H is raised the inactivation proceeds more rapidly and more dilute S.D.S. can be used, but the effect is complicated by the inactivation caused by the heating or alkali alone. At either room temperature or 37° the products of alkaline inactivation of tobacco mosaic virus remain soluble in the presence of small amounts of salts, although they are readily coagulated by strong salt solutions. At 55° or higher, however, the protein precipitates as it is liberated from combination with the nucleic acid unless the solution is more alkaline than *p*H 11. In tests in which tobacco mosaic virus at *p*H 10-14 was heated for varying periods at 55°, the falls in infectivity and serological activity were found to be proportional to the weight of protein precipitated, and this weight when plotted against the log of time gave a straight line. After 160 min. the preparation was completely inactivated, and 87 % of the starting material was precipitated as a carbohydrate-free protein, a figure agreeing well with the expected content of protein free from nucleic acid. As our results on the increase in the rate of alkaline inactivation of tobacco mosaic virus by heat, which may equally well be regarded as a reduction in the thermal inactivation point by increasing *p*H, agree in all essentials with those of Lauffer & Price [1940] they need not be given in detail. This effect is not confined to tobacco mosaic virus,

for at 55° both potato virus "X" and tomato bushy stunt virus are rapidly inactivated at *p*H values that are without appreciable effect at room temperature.

Table 1. *The effect of sodium dodecyl sulphate on tobacco mosaic virus*

Sample	Concentration of S.D.S.	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
A	0	1/1,280,000	6	1
B	0·043%	1/640,000	2	1
C	0·073%	1/160,000	0	0
D	0·143%	1/10,000	0	0
E	0·073% neutralized	1/1,280,000	104	47
F	0 control	1/2,560,000	205	95

Samples A, B, C, and D were 0·3% solutions of tobacco mosaic virus in *M*/15 glycine : NaCl buffer at *p*H 9·3 exposed for 1 hr. at 55° to the concentration of S.D.S. stated. They were then taken to *p*H 6 with HCl and phosphate buffer and diluted for testing. In E the virus was added to a neutralized and diluted buffer mixture containing as much S.D.S. as C, and in F virus was added to neutralized buffer only.

Table 1 shows the effect of S.D.S. on tobacco mosaic virus at *p*H 9·3 and 55°. In the absence of S.D.S. there is a definite fall in the infectivity of the preparation that is not paralleled by a similar fall in serological titre. A similar dissociation of these two properties with tobacco mosaic virus can be produced by oxidizing agents, nitrous acid, formaldehyde and irradiation with X-rays or ultra-violet [Bawden & Pirie, 1937; Stanley, 1936], and with tomato bushy stunt virus by alkali [Bawden & Pirie, 1938, 2]. The conditions of *p*H and temperature within which there is complete, or almost complete, loss of infectivity with tobacco mosaic virus without great loss of serological activity are narrowly circumscribed, and it is seldom that such a definite difference as that between samples A and E in Table 1 is obtained. Samples which lose their infectivity but not their serological activity in this manner retain their ability to show anisotropy of flow. With slightly more severe treatment, however, secondary changes rapidly follow those that lead only to a loss of infectivity, and the preparations lose both their serological activity and anisotropy of flow.

It is clear that the action of alkali on tobacco mosaic virus is complex. Eriksson-Quensel & Svedberg [1936] and Wyckoff [1937] found that after exposure to alkali between *p*H 9 and 11 tobacco mosaic virus was split into a number of products of smaller molecular weight and that the extent of the disintegration depended on the *p*H. These workers made no measurements on the activities of their preparations after these treatments, but Best [1936] found that both purified and crude preparations were 50% inactivated by 12 hr. exposure to *p*H 8·2 at 17°. The purified preparations that we have used have been much more resistant to alkali than this. Table 2 shows the results of one of our experiments. There was no difference between the control and a sample that had been held at *p*H 8·58 for 24 hr. at 20°, and only above *p*H 10 was there great inactivation. The treatment at *p*H 10·5 again shows the dissociation of infectivity from serological activity. The treatment at *p*H 9·3 shows an effect which we have frequently obtained in these alkali tests, an apparent increase in the infectivity by gentle treatment. This activation can be regarded as a partial reversal of the fall in infectivity that is produced by rigorous chemical purification of the virus. This is believed to be caused by the linear aggregation of

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Table 2. *The effect of alkali on tobacco mosaic virus*

*p*H	Time	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
11	24 hr.	1/40,000	0	0
10·5	24 hr.	1/3,000,000	12	2
9·3	24 hr.	1/4,000,000	106	21
8·5	24 hr.	1/4,000,000	74	9
8·5	5 min.	1/4,000,000	65	11
	Control	1/4,000,000	73	10

In each sample 0·1 ml. of 5·3% tobacco mosaic virus solution was added to 1 ml. of *M*/10 glycine : NaCl buffer at the *p*H stated. 0·08 and 0·06 ml. of *N*/10 NaOH were added to the first and second, for interpolation on the titration curve of tobacco mosaic virus shows that these amounts are necessary to bring 5·3 mg. of virus to *p*H 11 and *p*H 10·5 respectively. After the time stated, each was neutralized with acetic acid and diluted for testing. In the control, virus was added to 1 ml. of *p*H 8·5 buffer previously neutralized with acetic acid.

virus particles, and the simplest explanation of the reactivation is that the alkali disaggregates the long rods. Thus at least three successive effects of alkali on tobacco mosaic virus can be detected. First, there is a disaggregation of the purified material, secondly, a change within the particles that renders them non-infective without destroying their structure or serological properties and thirdly, a disruption of the particles that leads to a loss of all characteristic properties. The course of the third stage is also influenced by the temperature, for at low temperatures the products of denaturation remain soluble, whereas at high temperatures they coagulate.

The action of S.D.S. in the presence of alkali differs from the action of alkali alone. For example, in the presence of sufficient S.D.S. to cause appreciable inactivation of tobacco mosaic virus there is no precipitate of denatured protein such as would be produced by the action of alkali and temperature alone. Thus, in the treatments described in Table 1, although A contained a precipitate, B and C were only opalescent and D was perfectly clear. In attempts to gain further information on the antigenic constitution of tobacco mosaic virus, rabbits were injected with the phosphorus-free protein obtained by treating the virus with S.D.S., but the serum failed to react with either intact virus or that treated with S.D.S.

Tomato bushy stunt virus is even less readily attacked by S.D.S. than tobacco mosaic virus, and in neutral solution it has never been completely inactivated in any of our experiments. This may be because an equilibrium is established, but we have no definite information on this. In solutions containing 4·4% S.D.S. and 2·6% bushy stunt virus and 3·5% $Na_2HPO_4,12H_2O$ precipitates separate almost immediately, the precipitates appearing amorphous under the microscope. Like the similar, though shimmering, precipitates that separate with tobacco mosaic virus, these dissolve in about 30 min. at 37°. In these conditions a second precipitate, apparently a complex of S.D.S. and modified protein, separates after a few hours; this precipitate, like that given by the unchanged virus with ammonium sulphate, dissolves when the fluid is cooled. After 20 hr. at 37°, a dialysed sample still contains about 10% of unchanged virus. This mixture can be partially separated either by centrifuging at 16,000 r.p.m. or by ammonium sulphate precipitation, for the inactive products formed by the action are not sedimented by a few hours' centrifuging at this speed and they are more easily precipitated by ammonium sulphate than the virus, especially at 0°. The inactive

protein that can be separated by the addition of ammonium sulphate to slightly alkaline solutions is almost free from phosphorus and carbohydrate, and nucleic acid can be precipitated from the supernatant fluids by the addition of acid.

With tomato bushy stunt virus it is much easier to destroy infectivity without destroying serological activity by means of alkali than it is with tobacco mosaic virus. We have previously described [Bawden & Pirie, 1938, 2] this effect when bushy stunt virus is exposed to *p*H 11 at 18°, but at higher temperatures the effect is more definite and can be produced in less alkaline solutions. In one experiment exposure for 30 min. at 55° at *p*H 9 and 9·7 in *M*/10 glycine buffer gave no change in the serological titre, although at a dilution of 10^{-4} g. of protein per ml. the samples gave 3 and 0 lesions respectively, whereas the control mixed with neutralized buffer at 55° gave 720 lesions. From preparations treated in this manner rhombic dodecahedral crystals can be obtained by slow precipitation with ammonium sulphate that are apparently identical with those formed by fully active virus. This non-infective crystalline material is indistinguishable, by the chemical, physical and serological tests that we have applied, from the virus isolated directly from infective sap, but careful solubility measurements have not yet been made. The existence of a protein that is not infective although it closely resembles the active virus in its properties suggests the possibility that such a material may often be a contaminant of purified virus preparations as normally prepared. This is especially likely as a similar type of inactivation seems to proceed in crude infective sap, although here, as the *p*H is usually around 5·5, the effect cannot be attributed to alkaline inactivation. Smith [1937] observed that infective sap from bushy stunt plants rapidly lost its infectivity and was inactive within a month. We have confirmed this observation but have found in striking contrast that purified preparations do not lose their infectivity in this manner. After more than a year at room temperature the infectivity of purified preparations of this virus has not been found to be significantly reduced. Samples of infective sap have been tested for serological activity and infectivity over periods of time, and although in all the infectivity has been found to fall off rapidly the serological activity has remained constant. Samples of infective sap kept over a year at room temperature and which had been non-infective for a year were still found to be serologically active, and from them we isolated a non-infective crystalline nucleoprotein by the methods used for normal virus isolation. This material was found in the same quantities and gave the same serological titre as fully active virus preparations. The possibility that preparations may consist of mixtures of infective and non-infective nucleoprotein may explain the fact that different preparations give remarkably constant serological titres but differ in their infectivities.

Bushy stunt virus preparations lose their activity in alkaline solutions more rapidly in the presence of S.D.S. and inactivation occurs at lower *p*H values. The action is also not restricted to one that merely destroys infectivity, for the nucleic acid is split off from the non-infective protein and the preparation loses its serological activity. In one test in which a 1% solution of the virus was exposed for 160 min. to 2·6% S.D.S. at *p*H 9 and 18°, the preparation lost its infectivity but could be split into various fractions after dialysing for a week at 0°. The addition of 1/8 vol. of saturated ammonium sulphate solution precipitated a nucleic acid-free protein, which represented 75% of the original weight of virus, was amorphous and did not react with virus antiserum. With more ammonium sulphate there was slow crystallization at 0° of a non-infective, though serologically active, nucleoprotein similar to that already described. The yield of this was 12% of the starting material.

The absorption spectrum of tomato bushy stunt virus has a pronounced maximum at 260 mμ. After partial inactivation by S.D.S. the curve has the same shape but it lies below the curve for fully active virus. Thus a 2 cm. layer of a 0·016% solution of the virus transmitted 1/100 of the incident light at 260 mμ ($\log_{10} I_0/I = 2$), whereas after treatment with S.D.S. so that half of the virus was destroyed the transmission was doubled at 260 mμ ($\log_{10} I_0/I = 1{\cdot}7$). We have described a similar result during the inactivation of potato virus "X" and tobacco mosaic virus by urea, during which the nucleic acids are separated from the proteins. In the inactivation of bushy stunt virus by urea, on the other hand, the nucleic acid is not liberated, and there is no such decrease in opacity.

Urethane

The pharmacological action of the urethanes led naturally to their use in the study of enzyme systems, and Warburg & Wiesel [1912] pointed out that there was a correlation between the ability of a narcotic to give a precipitate with yeast maceration juice and its inhibitory action on the respiration of various tissues. Meyerhof [1918] confirmed these observations, and found that the presence of salt was necessary for the formation of a precipitate by urethanes in protein solutions. Hopkins [1930] measured the rate of denaturation of egg albumin in urethane and the slower rate of denaturation of sheep serum proteins, and he commented on the fact that the solvent powers of urethane were less than those of urea. On the other hand, Jirgensons [1936] found that 25% methylurethane impeded the precipitation of casein by salts.

In neutral solution strong ethylurethane rapidly denatures tobacco mosaic virus and is a solvent for the denatured products even in the presence of salts. At *p*H 7 the anisotropy of flow of virus solutions disappears after a few minutes in 28–40% solutions of urethane and the solutions become perfectly clear, although on dilution a precipitate develops if traces of salt are present. With more dilute urethane the anisotropy of flow does not quite disappear, the loss of infectivity and serological activity is only partial and the inactivated virus precipitates although it can readily be dissolved by the addition of more urethane. The rate of inactivation of tobacco mosaic virus by urea is greatly increased by cooling below 20°, but inactivation by urethane is not. After 48 hr. at 0° a 1·5*M* solution of urethane in *M*/30 phosphate buffer of *p*H 7 containing 0·6 g. of virus per litre gave a serological titre of 1/640,000 and an average number of lesions per leaf of 68 and 5 at dilutions of 10^{-4} and 10^{-5} respectively. After the same time at 0° the preparation gave the same titre and the lesions were 45 and 5, whereas the control gave a titre of 1/1,280,000 and 86 and 19 lesions per leaf.

Table 3. *The effect of urethane on potato virus "X".*

Concentration of urethane *M*	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
0·5	1/1,280,000	171	52
1·0	1/640,000	137	41
2·26	No ppt. at 1/10,000	0	0
Control	1/1,280,000	193	64

In each sample a 0·08% solution of potato virus "X" in *M*/50 *p*H 7 phosphate buffer was exposed for 24 hr. at 18° to the concentration of urethane stated. They were then diluted with 3 vol. of water and diluted further for testing after 36 hr. For the control an amount of urethane equal to that in the *M* sample was added immediately before diluting and testing.

The solubility of urethane at 0° is too small to permit experiments under conditions in which inactivation is more complete.

Tomato bushy stunt virus is much more resistant to urethane than is tobacco mosaic virus; 22 hr. exposure at 23° altered neither the serological titre nor the infectivity of a 0·14% virus solution in 0·023 *M* phosphate buffer at *p*H 7·8. Potato virus "X", however, is more susceptible and Table 3 shows the inactivation of this virus by urethane. The denatured virus was not held in solution by 2·26 *M* urethane and at that concentration the anisotropy of flow disappeared almost immediately and a precipitate separated. At the lower concentrations there was no precipitation and only a reduction in the anisotropy of flow.

Guanidine

Guanidine, like many of the other agents used in these experiments, affects proteins in a number of different ways. Petrumkin & Petrumkin [1927; 1928] found that gelatin and a mixture of denatured proteins from brain combined with guanidine in alkaline solution, and Grynberg [1933] measured the amount of combination under various conditions of *p*H, salt and guanidine concentration, with casein, gelatin, egg albumin and a globulin. Svedberg [1937] studied the dispersive action of guanidine on haemocyanin by means of the ultracentrifuge, and its action on myosin was studied by Edsall & Mehl [1940] who followed the loss of anisotropy of flow and viscosity. Denaturation with the production of —SH groups was found with egg albumin [Greenstein, 1938], excelsin, edestin and globin [Greenstein, 1939], tobacco mosaic virus [Stanley & Lauffer, 1939] and with myosin [Greenstein & Edsall, 1940]. Where comparative tests have been made guanidine has been found to denature at lower concentrations than other agents such as urea, and to produce a larger number of —SH groups. Greenstein [1938; 1939] found with both urea and guanidine that doubling the concentration, within the critical range, more than doubles the amount of —SH produced. This result parallels the well-known effects of variations in the concentration of disinfectants and agrees with the effects of variations in the concentration of the various virus-inactivating agents described in this paper.

Neutral solutions of guanidine hydrochloride, if more concentrated than 0·2 *M*, precipitate tobacco mosaic virus from solution. The precipitate can be washed with guanidine solution, but readily dissolves in water. Like the precipitate produced when clupein is added [Bawden & Pirie, 1937], it has a fibrous structure, but unlike the clupein precipitate it is not dissolved by the addition of small amounts of salt. There is no loss of activity during this precipitation provided that solutions less than *M* are used, but in more concentrated solutions there is some denaturation and then the precipitate produced is amorphous. In guanidine solutions more concentrated than 2·5 *M* the active virus does not precipitate, but there is rapid inactivation and a precipitate of denatured protein separates. The course of inactivation of tobacco mosaic virus by strong guanidine and its precipitation by dilute guanidine are illustrated in Table 4.

Tomato bushy stunt virus differs from tobacco mosaic virus in its behaviour towards guanidine. It does not precipitate with dilute solutions and concentrated solutions retain the denatured virus in solution. For example, virus exposed to 3·3 *M* guanidine did not precipitate on dilution. After inactivation by exposure for 21 hr. at 23° to 1·3 *M* guanidine, however, a precipitate separated on dilution. It is clear that with these two viruses, as with the proteins that Greenstein has studied, guanidine is effective at lower concentrations than urea.

Table 4. *The effect of guanidine on tobacco mosaic virus*

Treatment	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
A. 24 hr. in 2·7 *M* guanidine hydrochloride	No ppt. at 1/10,000	0	0
B. 24 hr. in 2·25 *M* guanidine hydrochloride	1/80,000	36	4
C. 5 min. in 0·68 *M* guanidine hydrochloride, then centrifuged:			
(1) Precipitate	1/640,000	200	80
(2) Supernatant	No ppt. at 1/10,000	2	0
D. Control; guanidine hydrochloride added after dilution	1/640,000	209	85
E. Control; no guanidine hydrochloride	1/640,000	228	99

All the tests were made at 17° with 1 ml. samples of a 1·1% solution of tobacco mosaic virus in 0·075 *M* phosphate buffer at *p*H 7. To A and B were added 0·4 and 0·2 ml. of 3·38 *M* guanidine hydrochloride, to C 0·3 ml. of water and 0·2 ml. of guanidine, and to D 2·2 ml. of water and 0·2 ml. of guanidine. After the stated time each was made up to 2·5 ml. with water, and diluted further for testing 36 hr. later.

Pyridine, picoline, lutidine, aniline and nicotine

The action of aqueous pyridine on proteins has been but little studied, although it has long been known to dissolve some [Levites, 1911] and to make gelatin swell even when neutralized [Fischer & Sykes, 1915]. Spiro [1900] observed that dilute pyridine lowered the temperature of coagulation of proteins and changes in haemoglobin [Jirgensons, 1936] and tobacco mosaic virus [Bawden & Pirie, 1937] have been brought about by it.

The neutral pyridine used in our tests was made by diluting 8 ml. of pyridine and 0·5 ml. of *N* HCl to 25 ml. with water. Neutral picoline was made in the same way. In 3 *M* pyridine tobacco mosaic virus and potato virus "X" lose their anisotropy of flow almost immediately and give clear solutions from which small, sticky precipitates separate after 24 hr. In more concentrated pyridine the inactivated viruses do not precipitate, while in more dilute pyridine the anisotropy of flow disappears slowly and there is an increase in opalescence leading finally to precipitation. The behaviours of these two viruses in picoline are similar to those in pyridine, but picoline has a slightly greater inactivating power. Tables 5 and 6 illustrate the inactivating effects of pyridine and picoline on tobacco mosaic virus. Potato virus "X" is inactivated under much the same conditions as tobacco mosaic virus, but tomato bushy stunt virus is more resistant. Thus, 20 hr. exposure at 18° to either 2·4 *M* pyridine or 1·3 *M* picoline

Table 5. *The effect of pyridine on tobacco mosaic virus*

Concentration of pyridine *M*	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
1·33	No ppt. at 1/10,000	0	0
0·625	1/640,000	101	54
Control	1/640,000	118	72

Tobacco mosaic virus solutions containing 0·052% virus and *M*/30 *p*H 6·95 phosphate buffer were exposed to the concentration of pyridine stated for 24 hr. at 16°. They were then diluted so that the pyridine was 0·312 *M* and were further diluted for testing 36 hr. later. In the control 0·312 *M* pyridine was added to the virus immediately before diluting and testing.

Table 6. *The effect of picoline on tobacco mosaic virus*

Concentration of picoline *M*	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
0·6	1/80,000	12	3
0·4	1/640,000	66	21
0·2	1/1,280,000	104	51
Control	1/2,560,000	165	69

Each sample contained 1·2 mg. of tobacco mosaic virus and 0·1 ml. of *M*/10 *p*H 7 phosphate : borate buffer and 0·3 ml. of *M* picoline in a total volume of either 0·5, 0·75, 1·5 or 2·5 ml. The first three samples were kept 18 hr. at 18° when they were diluted to 2·5 ml., and after 36 hr. they were further diluted for testing. The picoline in the control was added immediately before diluting and testing.

had no effect on the serological titre of bushy stunt virus and only lowered the infectivity slightly. A commercial mixture of lutidines was also tested on tobacco mosaic virus. This was as good a precipitant as pyridine or picoline, i.e. precipitation was complete after 1 hr. in a 4% solution, but its solubility in water was insufficient to give concentrations great enough to have any solvent action on the denatured virus. Picoline, lutidine and pyridine all separate the nucleic acid from the protein in tobacco mosaic virus.

The protein-precipitating power of aniline has often been commented upon [e.g. Spiro, 1900], and it is therefore surprising that Lauffer [1938] found that exposure for 10 min. to concentrated mixtures of aniline and glycerol had no effect on the infectivity of tobacco mosaic virus. We have been unable to confirm Lauffer's result; 2 vol. of a 2·35% solution of purified tobacco mosaic virus were added to 98 vol. of a mixture of 48 parts of glycerol and 50 parts of aniline (one of the mixtures used by Lauffer), and samples were withdrawn and diluted for infectivity tests. Dilutions of the virus in water and in the already diluted aniline : glycerol mixture were used as controls. Table 7 shows the results from

Table 7. *The effect of a glycerol : aniline mixture on tobacco mosaic virus*

Time of contact	Average no. of lesions per leaf at 10^{-5}	Average no. of lesions per leaf at 10^{-6}
Diluted immediately	18	10
30 min.	10	6
90 min.	7	4
4 hr.	10	6
Virus added to diluted mixture	83	20
Virus diluted in buffer	84	19

Description in text.

one such experiment. It is apparent that contact of the virus with the glycerol : aniline mixture caused a great immediate reduction in infectivity, but the reduction did not increase greatly as the time of contact was extended. That the effect was one of the mixture on the host plant seems improbable, for at the dilution present in the inoculum there was no fall in infectivity. There is no obvious reason for the difference between Lauffer's results and our own, but the possibility that different strains of tobacco mosaic virus may differ in their resistances to inactivation must be remembered.

The birefringence of tobacco mosaic virus in neutral, salt-free solutions containing up to 20% of nicotine remains unaffected even after several days and

no precipitate separates. In the presence of salt, however, an opaque, fibrous precipitate separates after a few hours. On gentle shaking this precipitate disappears completely, leaving a fluid indistinguishable in appearance from a normal virus preparation. After a few hours the precipitate again appears and can again be dispersed by shaking; the process can apparently be repeated indefinitely over a period of weeks. A suitable mixture for demonstrating this phenomenon contains 0·4 % of virus, 4 % of NaCl and 7 % of nicotine neutralized with acetic acid, but other mixtures, e.g. those made from neutralized nicotine tartrate, work equally well. Similar, though rather less striking, results are obtained by dissolving tobacco mosaic virus in neutral *M* arginine hydrochloride. Exposure to this type of precipitating agent does not inactivate the virus, and 2 months' exposure to 15 % nicotine at *p*H 7 and 18° had no appreciable effect on the infectivity and serological activity. Fukushi [1930] found that 3 % nicotine caused partial inactivation of tobacco mosaic virus in 3 days, but as he was using crude infective sap there is no necessary contradiction between his results and ours.

There are two analogies for this type of reversible precipitation. The first is the birefringent liquid layer that separates from concentrated virus preparations [Bawden & Pirie, 1937], for by vigorous shaking this can be resuspended in the form of tactoids that are too small to be seen and have a refractive index too close to that of the surrounding medium to give a visible opacity. Best [1937] described a precipitate settling from an old sample of clarified, infective sap that may well be identical with the nicotine precipitate. This was also dispersed on shaking and reformed on standing undisturbed. In addition to the physico-chemical interest attached to precipitates of this type, their formation, especially in fluids with compositions similar to plant sap, may throw some light on the mechanism of formation of the characteristic inclusion bodies found in plants infected with certain viruses. The other systems that have been considered as models for this phenomenon [Bawden & Sheffield, 1939] bear less resemblance to normal cell contents than those described here.

Phenol, salicylic acid and benzoic acid

Runge [1834], the discoverer of phenol, found that it was a protein coagulant, and its power of inhibiting enzymes was stressed by Plugge [1872], who was also one of the first to use it as a disinfectant. Buchner & Hoffmann [1907] and Duchacek [1909] correlated this inhibitory power with the precipitation of protein. Innumerable studies with bacteria and animal viruses have shown that 5–10 % phenol is usually lethal. Henderson [1933] found that the stability of tobacco ringspot virus in crude sap was increased by the addition of 0·25 % phenol, and Stanley [1935] found that 1 % phenol had little effect on tobacco mosaic virus. Bawden [1935] found that 2 % phenol acting for 48 hr. at 1° had no effect on potato virus "X", whereas 3 % destroyed infectivity and either greatly reduced or destroyed serological activity.

In concentrated solutions phenol is usually a protein solvent, but the molecular weights found for proteins dissolved in this manner have been so low [Troensgaard & Schmidt, 1924; Cohn & Conant, 1926] that it is probable that they were dissociated. Measurements in the ultracentrifuge appear to confirm this dissociation [Lundgren, 1938]. The effects of salicylates appear to be similar to those of phenol. Dilute solutions precipitate proteins [von Furth, 1896] whereas strong solutions either dissolve proteins [Neuberg, 1916] or cause swelling [Stoeltzner, 1925]. Meissner & Wöhlisch [1937] found that fibrinogen solutions had a maximum opacity when they contained 12·5 % sodium salicylate,

which agrees well with the conclusions of Pauli & Weiss [1931] on the coagulation and solution of a number of proteins. Anson & Mirsky [1933] studied the reversible denaturation of methaemoglobin and found that 0·32 *M* salicylate caused 50% denaturation. Best [1940] found that dilute salicylate gave a birefringent precipitate with tobacco mosaic virus that was still active, whereas more concentrated solutions gave a precipitate of denatured inactive protein. In 0·5 *M* potassium salicylate a steady state of partial inactivation was set up, although inactivation was almost complete after a few hours at 30° in *M* solution. Tomato spotted wilt virus was much more susceptible, being completely inactivated in 1 hr. at 30° by 0·1 *M* salicylate.

Neuberg [1916] found that most of the aromatic acids had a solvent or "hydrotropic" action on proteins, but the changes that benzoic acid and its derivatives cause when they act on proteins have not been investigated. The swelling action of benzoate on pieces of dura mater was studied by Stoeltzner [1925] and Pauli & Weiss [1931], and Meissner & Wöhlisch [1937] used benzoates and hippurates as protein solvents.

Tables 8 and 9 illustrate the course of inactivation of tobacco mosaic virus and tomato bushy stunt virus by phenol. With these viruses, as with potato virus "X", in the critical range small changes in the phenol concentration have

Table 8. *The effect of phenol on tobacco mosaic virus*

Concentration of phenol *M*	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
0·416	No ppt. at 1/10,000	0	0
0·333	1/640,000	42	9
0·25	1/1,280,000	80	21
Control	1/1,280,000	75	22

0·47% solutions of tobacco mosaic virus were exposed to the concentration of phenol stated for 20 hr. at 23°, when they were diluted for testing. In the control the phenol was added immediately before diluting. The phenol solution was adjusted to *p*H 7 by the addition of NaOH.

Table 9. *The effect of phenol on tomato bushy stunt virus*

Concentration of phenol *M*	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
0·416	1/80,000	0	0
0·2	1/320,000	41	7
Control	1/640,000	74	19

0·16% solutions of tomato bushy stunt virus at *p*H 7 were exposed for 21 hr. at 23° to the stated concentrations of phenol, and then diluted and tested.

a large effect on the extent of the inactivation. In the absence of salt phenol does not give a precipitate with either of the viruses, but in the presence of salts a precipitate of denatured protein separates, the extent of the precipitation closely paralleling the loss of activity. As with other inactivating agents such as urea, the precipitate that separates from the inactivated tobacco mosaic virus is free from nucleic acid, whereas that from the inactivated tomato bushy stunt virus still has the nucleic acid attached.

The anisotropy of flow of tobacco mosaic virus preparations is destroyed instantly in 2 *M* sodium salicylate at 20° and in 1·4 *M* it disappears in 10–15 min. These inactive fluids remain quite clear unless they are diluted, when amorphous

precipitates separate. In more dilute salicylate the loss of anisotropy of flow is accompanied by increased opalescence, and in the range M to $0{\cdot}4\,M$ there is precipitation of amorphous protein within a few hours. Anisotropy of flow, infectivity and serological activity, although diminished, persist for some days at 18° in $0{\cdot}4\,M$ salicylate. As our results with salicylate are in good agreement with Best's [1940], they are not given in detail. Table 10 shows the effects of

Table 10. *The effect of sodium salicylate on tomato bushy stunt virus*

Concentration of salicylate M	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
1	No ppt. at 1/10,000	0	0
0·5	1/320,000	49	10
0·33	1/320,000	66	34
0·2	1/320,000	70	27
Control	1/320,000	79	37

For the first four samples 0·1 ml. of 1% tomato bushy stunt virus was mixed with 0·1 ml. of neutralized $2\,M$ sodium salicylate; before the addition of the salicylate, 0·2 ml. of water was added to sample 2, 0·4 ml. to sample 3 and 0·8 ml. to sample 4. After 48 hr. at 23° the samples were diluted for testing. Salicylate was added to the control immediately before dilution and testing.

salicylate on tomato bushy stunt virus. Of the samples only that at M concentration gave any precipitate of denatured protein. In this experiment, as with others, there is evidence that $0{\cdot}5\,M$ salicylate may be affecting infectivity without destroying serological activity. At salicylate concentrations $> M$ inactivation occurs rapidly, but the material remains in solution. Salicylate has a much greater deleterious effect on plant leaves than any of the other agents we have used. Even at dilutions as great as $0{\cdot}08\,M$ it is necessary to wash the leaves immediately after inoculation to ensure that they shall not be damaged.

Sodium benzoate at *p*H 7 inactivates tobacco mosaic virus but only at higher concentrations than those necessary with salicylate; this inactivation is illustrated in Table 11. In the sample exposed to $2\,M$ benzoate, the anisotropy of flow disappeared in a few minutes and there was precipitation after 15 min., but in the others there was only slight loss of anisotropy of flow and increase in

Table 11. *The effect of sodium benzoate on tobacco mosaic virus*

Concentration of benzoate M	Serological titre	Average no. of lesions per leaf at 10^{-4}	Average no. of lesions per leaf at 10^{-5}
2	No ppt. at 1/10,000	0	0
1·2	1/320,000	64	16
0·8	1/640,000	90	25
Control	1/1,280,000	105	35
Control, mixed just before testing	1/1,280,000	114	37

For each of the three test samples 0·1 ml. of a 2·35% solution of tobacco mosaic virus was added to 0·5, 0·3 and 0·2 ml. of $2{\cdot}4\,M$ sodium benzoate solution adjusted to *p*H 7 with HCl. In addition samples 2 and 3 had had 0·2 and 0·3 ml. of water added. After 20 hr. at 22° they were all diluted to 2·35 ml. with water or benzoate to give a final concentration of $0{\cdot}5\,M$ benzoate, and they were further diluted for testing 36 hr. later. In one control virus was added to benzoate at $0{\cdot}5\,M$ and tested 36 hr. later; in the other, virus and benzoate were mixed immediately before diluting and testing.

opalescence. Sodium hippurate also inactivates tobacco mosaic virus, but still more slowly; 20 hr. exposure at 23° to 1·9*M* hippurate at *p*H 7 reduced the serological titre from $1/128 \times 10^4$ to $1/32 \times 10^4$, and the average number of lesions per leaf at a virus dilution of 10^{-4} from 105 to 58.

Discussion

Although tomato bushy stunt virus is more susceptible to physical changes such as freezing and drying than either tobacco mosaic virus or potato virus "X", it is more resistant to the inactivating effects of the organic agents used in our experiments. Also, its denaturation often takes a different course, for whereas the commonest effect with the anisotropic viruses is the separation of nucleic acid from the protein, this is unusual with tomato bushy stunt virus. Slight changes within the particle, leading to loss of infectivity without denaturation, are also commoner with bushy stunt virus than with the others.

The number of substances that might profitably have been tested in a survey of the organic agents that inactivate plant viruses is very large, and in this work it was possible to include relatively few. These also have been tested under limited conditions, although our more detailed study of the inactivating effects of urea suggests that many factors such as *p*H or temperature may greatly influence inactivation, and in other conditions it is possible that their actions might be different. Apart from agents that can be regarded as oxidizing agents, acids or alkalis, Spiro, Neuberg, Hopkins, Stanley and Greenstein have, in the papers already quoted, examined the effects of over 100 substances on proteins. These cover a wide chemical range, and the most interesting substances cause irreversible changes in a few hours in neutral solution at room temperature at concentrations below 4*M*. If this limit is imposed, the usual solvents, such as alcohol and acetone, whose precipitating and inactivating actions are well known even though little understood, are excluded except when they are acting on abnormally unstable proteins. Similarly urea, although so widely studied and used as a denaturant, appears to be one of the less active members of the group.

Spiro [1900; 1904], who made the first attempts to systematize the scattered observations of earlier workers, suggested that some of the agents that impede coagulation by heat do so by acting as bases, whereas others, notably the mustard oils, form soluble complexes with proteins. Several authors have adopted the second suggestion, and we have pointed out [Bawden & Pirie, 1940] that a reversible ordered association between urea and proteins is compatible with the observations that have been made on urea : protein systems. Steinhardt [1938] has suggested that if this association occurs at centres normally concerned in the formation of cross linkages between peptide chains in proteins and involves the breaking of the linkage there will, on the removal of the denaturing agent, be a greater tendency for random reformation of cross linkages the larger the number of cross linkages that has been broken. The nature of the cross linkages in proteins is still uncertain, but this picture of their reversible breakage to an extent determined by the concentration of the denaturing agent and with a corresponding change in the probability of their reformation in the original pattern seems to us a reasonably satisfactory interpretation of the observed results. On this picture the efficiency of a denaturating agent depends on its ability to form associations with and to break the normal cross linkages.

At present it is impossible to predict the action of a substance on proteins in solution from a knowledge of its physical and chemical properties, although attempts have been made to correlate the action with changes in surface tension

or dielectric constant. Thus Jirgensons [1936] stressed the significance of the dielectric constant, and showed that the concentration of a substance required to produce maximum opacity in a salt-free colloidal solution was proportional to the dielectric constant. This type of measurement, however, is complicated by the fact that it not only measures the ability of a substance to precipitate or denature a protein but also measures the ability of stronger solutions to act as protein solvents. Steinhardt [1938] concluded that there was no such proportionality, for he found that some active amides raised the dielectric constant whereas others lowered it. Hopkins [1930] drew the following conclusions from his study of denaturation of proteins by nitrogenous substances. "An amide structure is apparently necessary, but in certain relations its activity is lost. Among the ureas, mono-alkyl substitution, or unsymmetrical di-alkyl substitution, leaves the activity qualitatively intact. Symmetrical di-alkyl ureas, on the other hand, are inactive; one amino group must apparently remain unsubstituted. To judge from the case of acetyl urea, however, mono-acetyl substitution removes the activity. In biuret, allantoin and semicarbazide activity is also lost. Acid amides (acetamide and formamide) are active, but all amino-acids tried were without effect and likewise asparagine." However, as there seems to be no good reason to distinguish between the denaturing effects of nitrogenous substances and of non-nitrogenous substances such as benzoate, it is necessary greatly to extend the field in which any useful generalization must be valid. A survey of the literature and our own observations on some 15 substances suggests that the stability of a protein such as tobacco mosaic virus in a $4M$ solution of an organic substance is a more reasonable matter for comment than its instability in such an environment.

Summary

The effects of alkali and of 15 simple organic substances on tobacco mosaic virus and tomato bushy stunt virus are described. Some experiments with potato virus "X" are also included. Bushy stunt virus is the most resistant to denaturation and potato virus "X" the least. The effects of alkali on tobacco mosaic virus are complex; gentle treatment may increase infectivity, slightly more severe treatment causes loss of infectivity but not loss of serological activity, and more severe treatment causes loss of all characteristic properties. With bushy stunt virus inactivation without loss of serological activity occurs over a wider pH range, and crystalline non-infective preparations can be made from alkali-treated material. Apparently similar crystalline and non-infective preparations can be isolated from expressed sap allowed to age for some months. In the presence of alkali, sodium dodecyl sulphate readily destroys all the viruses, separating the nucleic acid from the proteins. With the exception of nicotine and arginine, which form with tobacco mosaic reversible, fibrous precipitates, all the substances we have tested at concentrations below $4M$ inactivate the viruses in neutral solution. Dilute solutions of these agents are often precipitants whereas concentrated ones dissolve the products of denaturation. Inactivation of tobacco mosaic virus and potato virus "X" is usually accompanied by the separation of the nucleic acid from the protein, but inactivation of bushy stunt virus is not.

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152. THE ISOLATION OF α-OESTRADIOL AND OESTRONE FROM HORSE TESTES

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(*Received 11 July 1940*)

ZONDEK [1934] reported the preparation of crude alcoholic extracts of horse testes which possessed a marked oestrogenic potency. Extracts obtained in this way were three hundred times as active oestrogenically as those prepared from a similar weight of mares' ovaries. The high oestrogenic potency of horse testes extracts has been confirmed by later workers. The present investigation was undertaken in order to isolate and identify the oestrogens responsible.

A crude alcoholic extract from 28 kg. of horse testes was generously placed at the disposal of the author by Dr A. S. Parkes. This extract was concentrated, *in vacuo*, the acetone-insoluble material was removed and the acetone-soluble fraction was saponified by refluxing with alcoholic NaOH. The greater part of the oestrogenic activity of the initial concentrate appeared in the alkali-soluble, saponifiable material. That part carried over into the non-saponifiable fraction was recovered by solvent partition. Following the partitioning of the saponifiable fraction between suitable solvents, the concentrate obtained was dissolved in NaOH and the *p*H was adjusted to 9·0. Ether extraction gave a "phenolic" fraction, which on further solvent partition gave 112 mg. of "phenols" possessing practically all the oestrogenic activity of the original "saps". These "phenols" were separated into ketones and non-ketones with Girard's reagent-*T*.

Oestrone was isolated from the ketonic material as the 3:5-dinitrobenzoate (M.P. 188–191° alone or 188–192° when mixed with authentic oestrone 3:5-dinitrobenzoate of M.P. 193–194°). The parent substance (M.P. 238–245°) had the same order of biological activity as authentic oestrone.

α-Oestradiol was isolated from the non-ketones, following digitonin precipitation, as its di-α-naphthoate which melted at 194° alone or when mixed with authentic α-oestradiol di-α-naphthoate (M.P. 194°).

EXPERIMENTAL

All M.P.s are uncorrected. Analyses are by G. Weiler, Oxford.

Bioassays. These were carried out by Dr C. W. Emmens, National Institute for Medical Research, Hampstead, without whose generous co-operation it would have been impossible to have undertaken the investigation. Except for those cases where a group assay is indicated, the dilution method of assay [Beall, 1940] was used. The results were interpreted in terms of oestrone.

Colorimetric assays. When the concentrates had been purified sufficiently their oestrogenic content was estimated by the colorimetric method of Kober [1938]. The colour obtained was read photometrically in an ordinary colorimeter. This was done by placing an Ilford grey screen (0·5 density) against the left-hand

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plunger of the colorimeter and the solution to be read in a micro-cup under the right-hand plunger. An Ilford spectral blue-green filter was placed over the eye-piece and the depth of solution required to give the same intensity of illumination as the standard grey screen was determined [cf. King *et al.* 1937]. Different batches of the β-naphthol reagent gave reproducible results with the same amount of oestrone. A standard curve, using known amounts of oestrone, was accordingly established and the values for the test solutions were read from it.

Preparation of the initial concentrate

The horse testes were minced and extracted in the cold with a relatively large volume of industrial alcohol (10 l./kg.). This extraction was repeated 3 times. The alcohol was decanted off and removed *in vacuo*. The residue obtained was then extracted 4 times with 12 l. portions of boiling acetone. After keeping the acetone solution several days in the ice box the supernatant liquid was decanted and taken to dryness. The residue weighed 387 g.

Concentration of the oestrogenic materials

The acetone-soluble material was saponified by refluxing for 1 hr. with 1 l. of 10% alcoholic NaOH and was separated in the usual way with ether into saponifiable and non-saponifiable fractions. Difficulties due to the formation of emulsions were overcome by the addition of alcohol. The aqueous alkaline solution of "saps" was then acidified and ether-extracted, the ether extract being washed with 2·5% $NaHCO_3$ which removed a large amount of acidic material. In this way 75 g. of "non-saps", 67 g. of "bicarbonate-washed saps" and 108 g. of "acids" were obtained. Bioassays showed that 70% of the total oestrogenic activity of the initial concentrate was in the "saps", the other 30% being divided equally between the "non-saps" and "acids".

The "non-saps" were dissolved in 1 l. of toluene and washed 6 times with 200 ml. portions of *N* NaOH. In each case the aqueous phase, which was partially emulsified, was cleared by centrifuging. The aqueous washings were combined, acidified and ether-extracted, the residue from the ether being added to the "bicarbonate-washed saps". An attempt to wash the toluene phase with water resulted in a very stable emulsion, so it was taken to dryness under reduced pressure, alcohol being added at the final stages to prevent frothing. The residue was taken up in ether, acidified and washed well with water. The ether was taken to dryness, the residue being dissolved in 1 l. of light petroleum and extracted 10 times with 100 ml. portions of 90% MeOH. The alcoholic washings were taken to dryness under reduced pressure and the residue taken up in 1 l. of toluene. The resulting solution was washed 6 times with 100 ml. portions of *N* NaOH. These washings were saturated with CO_2 and ether-extracted. The residue from the ether extract was added to the "bicarbonate-washed saps". In this way all the oestrogenic activity of the "non-saps" was transferred to the "saps".

Attempts to recover the oestrogens in the "acid" fraction were unsuccessful owing to the formation of stable emulsions and this fraction was discarded.

The "saps" were dissolved in 1 l. of light petroleum and extracted 10 times with 100 ml. portions of 90% methyl alcohol. The alcoholic extracts were taken to dryness under reduced pressure and the residue was dissolved in 250 ml. of *N* NaOH. 20 g. of $NaHCO_3$ were added, then *N* HCl was cautiously run in to *p*H 9·0, with vigorous stirring to avoid loss of CO_2. The solution was then extracted 8 times with 250 ml. portions of ether which was taken to dryness to give a "phenolic" fraction. The residue from the light petroleum weighed 33 g.

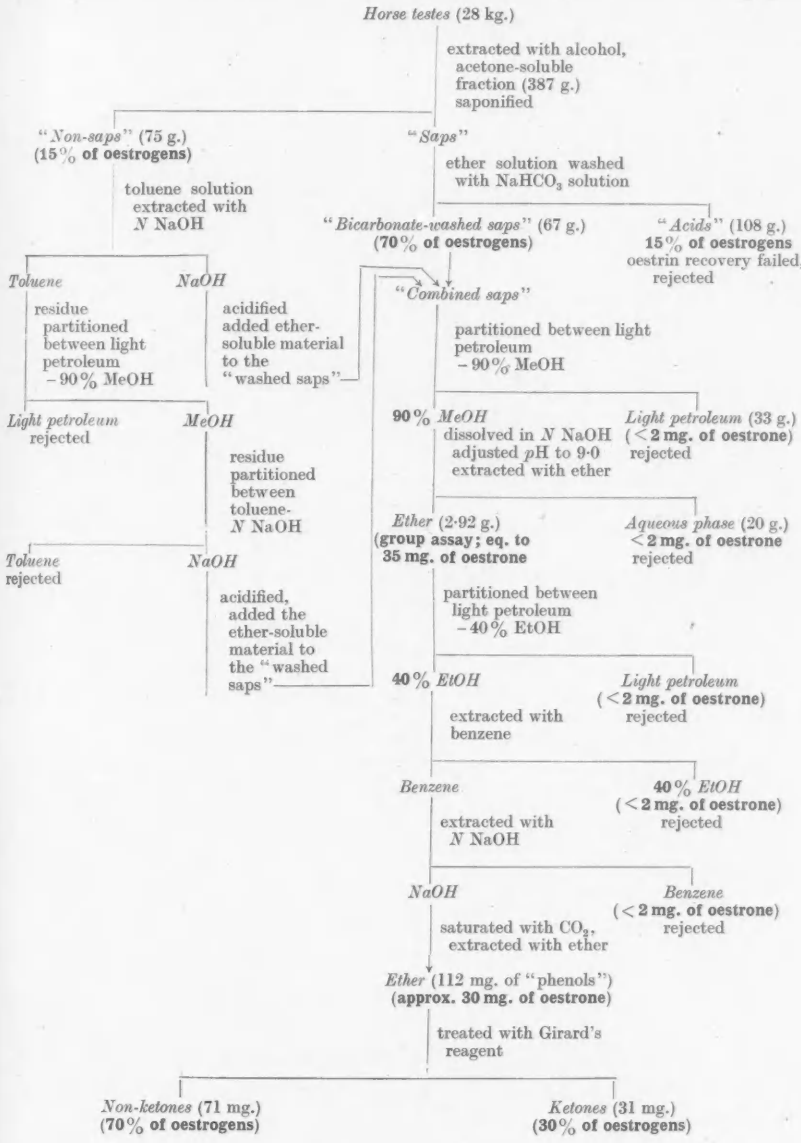
Horse testes (28 kg.)
extracted with alcohol, acetone-soluble fraction (387 g.) saponified
"*Non-saps*" (75 g.) (15% of oestrogens)
"*Saps*"
ether solution washed with $NaHCO_3$ solution
toluene solution extracted with *N* NaOH
"*Bicarbonate-washed saps*" (67 g.) (70% of oestrogens)
"*Acids*" (108 g.) 15% of oestrogens oestrin recovery failed, rejected
Toluene
NaOH
residue partitioned between light petroleum – 90% MeOH
acidified added ether-soluble material to the "washed saps"
"*Combined saps*"
partitioned between light petroleum – 90% MeOH
Light petroleum rejected
MeOH
90% *MeOH* dissolved in *N* NaOH adjusted *p*H to 9·0 extracted with ether
Light petroleum (33 g.) (< 2 mg. of oestrone) rejected
residue partitioned between toluene-*N* NaOH
Ether (2·92 g.) (group assay; eq. to 35 mg. of oestrone
Aqueous phase (20 g.) < 2 mg. of oestrone rejected
Toluene rejected
NaOH
acidified, added the ether-soluble material to the "washed saps"
partitioned between light petroleum – 40% EtOH
40% *EtOH*
Light petroleum (< 2 mg. of oestrone) rejected
extracted with benzene
Benzene
40% *EtOH* (< 2 mg. of oestrone) rejected
extracted with *N* NaOH
NaOH
Benzene (< 2 mg. of oestrone) rejected
saturated with CO_2, extracted with ether
Ether (112 mg. of "phenols") (approx. 30 mg. of oestrone)
treated with Girard's reagent
Non-ketones (71 mg.) (70% of oestrogens)
Ketones (31 mg.) (30% of oestrogens)

Fig. 1. Flow-sheet of the concentration of the oestrogens.

and contained less than 2 mg. of oestrogen, assayed as oestrone. The ether-soluble material obtained on acidifying the aqueous phase (of *p*H 9·0) weighed 21 g. and contained less than 2 mg. of oestrone. The "phenolic" fraction, which weighed only 2·92 g., had an oestrogenic activity, determined by a group assay, equivalent to 35 mg. of oestrone.

The "phenolic" fraction was dissolved in 100 ml. of light petroleum; 40 ml. of alcohol were added, followed by 60 ml. of water. An opalescent aqueous alcoholic phase separated after shaking. The light petroleum was re-extracted 9 times with 50 ml. portions of 40% alcohol. The combined aqueous alcoholic extracts were then extracted 6 times with 70 ml. portions of benzene which were in turn extracted 6 times with 50 ml. portions of *N* NaOH. The alkaline extracts were saturated with CO_2 till acid to phenolphthalein and ether-extracted. The residue from the ether extract (the "phenols") weighed 112 mg. and contained practically all the oestrogenic activity of the "phenolic" fraction.

The "phenols" were next separated into ketonic and non-ketonic fractions, using Girard's reagent-*T* by the method used in the isolation of oestrone from ox adrenals [Beall, 1940]. Bioassays showed that 70% of the oestrogens were concentrated in the non-ketones and 30% in the ketones. A summary of the partition process is given in Fig. 1.

Isolation of oestrone 3:5-dinitrobenzoate

The ketonic material (31 mg.), which was partially crystalline, was sublimed at 170°/0·05 mm. The sublimate obtained (14 mg.) was crystalline but contained some gum, so it was recrystallized from aqueous alcohol. This gave 3·7 mg. of crystals melting at 238–245°. A bioassay using a group of 10 mice, each receiving 0·06 μg. of these crystals, gave a 10% response. The same response in the control group was elicited by 0·055 μg. of pure oestrone. Colorimetric assay gave results in agreement with those obtained with the same amounts of pure oestrone. The remainder of the crystals was converted into the 3:5-dinitrobenzoate by treatment with 3:5-dinitrobenzoyl chloride in pyridine. The product was crystallized from benzene-alcohol and gave crystals which melted at 188–191° alone or 188–192° when mixed with authentic oestrone 3:5-dinitrobenzoate (M.P. 193–194°). While attempting to sublime the remainder of the crystals *in vacuo* a failure of the pump occurred and the product resinified. Because of this loss of material no analytical figures could be obtained.

Isolation of α-oestradiol di-α-naphthoate

The non-ketones were dissolved in 2 ml. of alcohol and 100 mg. of digitonin in 4 ml. of 50% alcohol were added. The mixture was refluxed for 1 hr. and kept overnight at room temperature. The resulting precipitate was centrifuged, and washed twice by stirring with ether and recentrifuging. The aqueous alcoholic filtrate was diluted with water and ether-extracted, the ether being combined with the ether washings of the digitonin precipitate. The combined ether was washed well with water. Colorimetric assay showed this fraction to contain the equivalent of 3·2 mg. of oestrogens, expressed as oestrone.

The digitonin precipitate was dried, powdered, heated on the water bath for 30 min. with 1·5 ml. of dry pyridine and then poured into 40 ml. of dry ether. The precipitate was filtered off and washed well with ether. It was completely soluble in water, showing that decomposition of the digitonide was complete. The ether was washed with dilute HCl and then water and taken to dryness.

It gave 5 mg. of gum which, assayed colorimetrically, contained the equivalent of 3·7 mg. of oestrone. It was converted into the di-α-naphthoate by the method of MacCorquodale *et al.* [1936], glycine being used to remove the unused acid chloride. The product was taken up in acetone, an equal amount of alcohol was added and the solution was concentrated to 2 ml. A precipitate separated rapidly. After 2 hr. this was collected, redissolved in 2 ml. of acetone and the solvent evaporated under nitrogen, 3 ml. of alcohol being gradually added. The final volume was 2 ml. The resulting crystals weighed 4·5 mg. They softened at 192° and melted at 194° alone or when mixed with authentic α-oestradiol di-α-naphthoate (M.P. 194°). (Found: C, 82·2; H, 6·02%. Oestradiol di-α-naphthoate requires C, 82·7; H, 6·25%.)

Discussion

The "phenols" of the saponifiable material contained the equivalent of 35 mg. of oestrone, estimated by a group assay. Of this 30% (10 mg.) was concentrated in the ketonic fraction and the other 70% (25 mg.) in the non-ketones. By the method of bioassay used α-oestradiol had 4 times the potency of oestrone so that the non-ketones possessed an oestrogenic activity equivalent to 6 mg. of oestradiol. Colorimetric assays of the digitonin precipitate and alcoholic filtrate of this material gave values of 3·7 and 3·2 mg. respectively, a total of 6·9 mg., which is in close agreement with the bioassay results.

That only half the oestrogenic material in the non-ketones was precipitated by digitonin may be due to the fact that α-oestradiol requires a large excess of this reagent for complete precipitation. Unfortunately the alcoholic filtrate from the first precipitation was lost so this point could not be investigated.

On the assumption that the oestrogenic activities of the non-ketonic and ketonic fractions were due to α-oestradiol and oestrone respectively the horse testes examined contained 0·21 mg. of α-oestradiol and 0·36 mg. of oestrone per kg. of wet tissue. This is a much higher concentration than has been reported for other organs. This is illustrated in the data given in the accompanying table.

Organ	α-Oestradiol mg./kg.	Oestrone mg./kg.
Sow ovaries	0·014*	0·010*
Human placentae	0·038†	0·035*
Ox adrenals	—	0·008‡
Horse testes	0·210	0·360

* Westerfeld *et al.* [1938]. † Huffman *et al.* [1940]. ‡ Beall [1940].

The presence of α-oestradiol in horse testes is of interest since it has hitherto been isolated only from tissues and fluids of female origin. Further, this finding prevents the attachment of any idea of sex specificity to the occurrence of this hormone. Speculation as to its path of formation in the male species would be idle with our present limited knowledge of steroid metabolism.

Summary

Using 28 kg. of horse testes α-oestradiol has been isolated (0·21 mg./kg.) as its di-α-naphthoate and oestrone (0·36 mg./kg.) as its 3:5-dinitrobenzoate.

The concentrations of oestrone and of α-oestradiol in horse testes are much higher than the values reported for any other tissue to date.

The author wishes to express his gratitude to Dr A. S. Parkes, National Institute for Medical Research, for so generously contributing the material for

this investigation and for his general interest in the problem. Thanks are also due to Mr W. Crisp of the same Institute for preparing the initial alcoholic concentrate.

Grateful acknowledgement is made to the Medical Research Council for a grant towards the cost of the investigation.

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153. THE COMPONENT ACIDS OF AN OX BONE MARROW FAT

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The composition of fat from interior cavities of the bones of the ox is of some interest in relation to that from other fatty deposits which have been much more completely studied. Actually, the information available on the component acids of bone marrow fats is very scanty and none appears to have been obtained with the aid of the ester-fractionation technique developed during the past twenty years. Analyses of many technical bone fats or "bone greases" have been given, but these are also restricted to the less detailed methods and, of course, the technical products known as bone greases include fat from tissues still adhering to the bones as well as the true bone (or marrow) fat and are thus of little biochemical interest.

The component acids of ox bone marrow fats were examined, however, many years ago by Nerking [1908] and by Eckart [1922]. Nerking reported that the acids of yellow marrow fat were palmitic 7·8, stearic 14·2 and oleic 78%, whilst those of the red marrow fat were palmitic 16·3, stearic 36·3 and oleic 47·4%. Eckart states that the average composition of the acids of ox bone fat is about 20% each of palmitic and stearic acids and 60% of unsaturated acids (oleic *ca.* 52% and linoleic *ca.* 8%). So far as can be ascertained, these records are based on the proportions and mean molecular weights of the acids from the lead salts of the mixed acids insoluble in ether, Eckart's values for oleic and linoleic acids being derived presumably from the iodine value of the acids of the ether-soluble lead salts. The high content of oleic acid and the very low content of palmitic acid given by Nerking for the yellow marrow fatty acids appear somewhat unlikely having regard to present day knowledge of the general composition of fats of most of the larger land animals.

An opportunity to examine the fatty acids in an authentic ox bone marrow fat by modern methods was recently given us by Dr R. A. Morton of this University, who had separated the unsaponifiable matter from the fat and kindly allowed us to make use of the residual fatty acids obtained during this operation. The fat had been prepared by mechanical removal of the yellow bone marrow from long ox bones which had been sawn into suitable sections. The fresh material was rendered and the melted fat decanted from any bone tissue residues. After the yellow marrow fat thus obtained had been saponified and unsaponifiable matter extracted as completely as possible from the aqueous soap solution, the latter was acidified and the resulting mixed fatty acids handed to us for examination. They had a mean molecular weight of 275·9 and I.V. 47·9.

The acids (100 g.) were submitted to a lead salt separation from alcohol by our usual procedure, the methyl esters of the two groups of acids from the respective insoluble and soluble lead salts then being fractionally distilled at 0·2 mm. pressure through an electrically-heated and packed fractionation column. The final numerical results of the investigation are summarized in Table 1.

Table 1. *Component acids of ox bone (yellow) marrow fat*

Acid	"Solid" acids (47·1%)	"Liquid" acids (52·9%)	Total	% (wt.)	% (mol.)
Lauric	—	0·1	0·1	0·1	0·1
Myristic	0·4	2·2	2·6	2·6	3·1
Palmitic	28·1	4·1	32·2	32·3	34·1
Stearic	15·5	—	15·5	15·5	14·7
Tetradecenoic	—	0·7	0·7	0·7	0·8
Hexadecenoic	—	3·0	3·0	3·0	3·2
Oleic	3·1	40·1	43·2	43·2	41·5
Octadecadienoic	—	2·5	2·5	2·6	2·5
Unsaponifiable	Trace	0·2	0·2	—	—

The component acids of this fat, as revealed by our analysis, are indistinguishable from those of a typical ox depot fat of the softer type (i.e. from the rump of the animal rather than the perinephric fat). The palmitic acid content of 34% falls near to the usual upper limit (33%) noted in ox depot fats [Hilditch & Longenecker, 1937], whilst the total C_{18} acids amount to 58·7%, compared with the normal limits of 60–65% given by these authors. The contribution (15%) of stearic acid to the total C_{18} acids is lower than, e.g. in most ox perinephric fats, but is of the order frequently observed in the softer fats or tallows from other adipose tissues of the animal. The proportions of the minor component acids (myristic, hexadecenoic, octadecadienoic etc.) are also typical of those observed in the average perinephric or rump fats of oxen; the amount of unsaturated C_{18} ester fractions obtained was too small, and their content of oleic acid too great, to permit us definitely to ascertain whether the octadecadienoic acid contained appreciable amounts of ordinary or seed-fat linoleic acid, or whether (as may be more probable in the light of results with other ox depot fatty acids [Hilditch & Longenecker, 1937]) it consisted of isomeric forms of this acid. It may be noted that traces of highly unsaturated C_{20-22} acids, usually present in ox depot fats, were not detected in the marrow fatty acids, but this may have been due to the comparatively small amount of material employed in the present analysis.

Whilst it is not desirable to formulate absolute conclusions from the results of investigation of a single specimen of ox bone marrow fat, it may perhaps be inferred that the component acids of this fat approximate much more closely to those of ox perinephric or rump fats than would appear from the data recorded by earlier workers.

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154. THE COMPONENT ACIDS AND GLYCERIDES OF SOME INDIAN OX DEPOT FATS

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An investigation of four specimens of depot fats from oxen from three widely separated districts in India has disclosed considerable differences between their composition and that of corresponding fats from English, American or Australian cattle. The Indian ox depot fats are relatively much less unsaturated (I.V. 26–31 as compared with 40–50) whilst the content of palmitic acid, in three out of the four instances examined, approaches 40% (mol.) of the total fatty acids (instead of the more usual $30 \pm 3\%$). In addition to component acid determinations on each of the fats, the component glycerides of the two fats with the highest and lowest contents of palmitic acid have therefore been studied by the procedure applied to an English ox depot fat by Hilditch & Paul [1938].

The Indian fats about to be described included the depot fats of a bullock and of a cow from Bombay, of a cow from Calicut (Malabar), and those of animals of both sexes from Calcutta. These were respectively obtained for us by the Director of Industries, Bombay, the Kerala Soap Institute, Calicut, and the Calcutta works of Messrs Lever Brothers and Unilever, Ltd., to all of whom we express our cordial thanks. These fats were prepared in India under qualified supervision, and we were informed that the diets of the animals concerned were of the following general nature: in the Bombay area oxen usually feed on grass, rice, jowar straw, wheat bran and cotton-seed, whilst in the Calicut (and probably also the Calcutta) area the main diet is dry paddy straw and fresh grass. The fats were taken from one or more depots (probably mainly the perinephric tissues) of one or more animals of the sex and locality mentioned. The Bombay fats contained over 1% of free fatty acid (as oleic) and were neutralized before their detailed examination was commenced; the free acidity of the Calicut fat was negligible.

The general analytical characteristics of the fats are given in Table 1.

Table 1. *Analytical characteristics of Indian ox depot fats*

	Sap. equiv.	I.V.	Free fatty acid % (as oleic)	Unsap. %	Fat M.P. (open tube)	Mixed acids setting-point
Bombay (cow), original	280·1	26·6	1·4			
Bombay (cow), neutralized	282·2	26·5	0·1	0·35	50·5°	47·6°
Bombay (bullock), original	282·0	26·1	2·4			
Bombay (bullock), neutralized	283·2	25·8	0·1	0·3	50·0°	48·5°
Calcutta, original	283·2	31·0	1·3	0·2	49·0°	48·4°
Calicut (cow), original	283·5	31·1	0·2	0·4	50·5°	48·7°

Component acids of the Indian ox depot fats

Each fat (*ca.* 150 g.) was converted into the mixed fatty acids, and the latter submitted to the usual lead salt separation from alcohol. The methyl esters of the "solid" acids were distilled from a Willstätter bulb, and those of the "liquid" acids through an electrically heated and packed column, following our customary procedure. From the analytical characteristics of the ester-fractions thus obtained the composition of the mixed fatty acids was calculated, with the final results given in Table 2.

Table 2. *Component acids of Indian ox depot fats*

	Bombay (cow) (neutralized)		Bombay (bullock) (neutralized)		Calcutta (original)		Calicut (cow) (original)	
Component acids:	%(wt.)	%(mol.)	%(wt.)	%(mol.)	%(wt.)	%mol.)	%(wt.)	%(mol.)
Saturated:								
Lauric	0·1	0·2	0·2	0·3	0·2	0·3	0·3	0·5
Myristic	4·5	5·2	3·7	4·4	2·4	2·8	3·1	3·7
Palmitic	41·4	43·3	37·1	39·1	36·8	38·8	32·9	34·7
Stearic	24·3	22·9	29·4	27·9	26·8	25·5	29·3	27·9
Arachidic	0·5	0·4	1·2	1·0	0·4	0·4	—	—
Unsaturated:								
Tetradecenoic	0·4	0·5	0·4	0·5	0·3	0·4	0·4	0·4
Hexadecenoic	1·3	1·4	1·0	1·0	2·1	2·2	1·5	1·6
Oleic	26·4	25·1	25·9	24·8	29·2	28·0	30·7	29·5
Octadecadienoic	1·0	0·9	0·9	0·9	0·9	0·9	1·3	1·3
C_{20-22} unsaturated	0·1	0·1	0·2	0·1	0·9	0·7	0·5	0·4

The most notable feature in the molar percentage data in Table 2 is the small content of unsaturated acids (27–33%) compared with that obtaining in all English, North and South American and Australian ox depot fats hitherto examined; in the latter the unsaturated acids have never fallen below 40%, and approach 50% in some instances. On the other hand, the proportion of stearic acid in the Indian fats, although substantial, does not exceed that observed in the more saturated (perinephric) ox depot fats from the other localities mentioned. In marked contrast to what has been uniformly observed in English, American and Australian depot fats, not only of oxen, but also of sheep and pigs [Hilditch, 1940], the deficiency in oleic acid in these Indian ox depot fats is made up not by a corresponding proportion of stearic acid but by an increased proportion of palmitic acid.

The contents of palmitic acid in three of these fats are, indeed, much larger than have hitherto been observed in any animal depot fat. Reviewing all the available detailed data for ox depot fat component acids, Hilditch & Longenecker [1937] were able to conclude that "the palmitic acid content of nearly all tallows which have been analysed lies within the relatively constant limits of 30 ($\pm$3)% (mol.)". This statement also applies to the numerous pig depot fats, both English and North American, which have been investigated in detail; whilst, over a much wider range of land animals (including birds) palmitic acid is roughly constant at 25–30% of the total component acids of their depot fats [cf. Hilditch, 1940]. An explanation of the higher proportions of this acid in the Indian ox depot fats cannot be suggested with certainty; it might be connected with species, diet or climate. There is nothing in the diet of these Indian oxen, as far as we are aware, to account for the unusually large percentages of palmitic acid; cottonseed, it is true, was mentioned as a constituent of the diet of the Bombay animals but, although cottonseed oil contains 20–25% of palmitic acid, inclusion of this oil in the diet of pigs causes a diminution in the palmitic

acid content of their depot fats [Ellis *et al.*, 1931; Bhattacharya & Hilditch, 1931]. It is equally unlikely that any species difference between the Asiatic and European oxen is concerned in this respect, since the depot fats of animals of many widely different families share the common feature of a content of 25–30 % of palmitic acid in their component acids. In this connexion it may be mentioned that Dhingra *et al.* [1938; 1939] found the component acids of North Indian goat depot fats to contain 27–28 % of palmitic acid.

On the other hand, the low content of unsaturated acids, i.e. the marked tendency towards saturation in the fats as a whole, may well be connected with the tropical temperatures in which these Indian cattle are reared. Further, it is possible that the simple oleic-stearic acid relationship observed in the depot fats of oxen, pigs etc. breaks down when the content of stearic acid reaches a certain point. At least it is suggestive that the molar proportion of stearic acid in the present series, and in other animal depot fats, reaches but has not been observed to exceed 27–28 % of the total fatty acids. It may be that, when the content of oleic acid falls, as in these cases, much below 40 %, the proportion of palmitic acid is supplemented so that stearic acid does not rise above a possibly limiting amount of 27–28 %. However this may be, the augmentation of the palmitic acid content and the limitation of that of stearic acid has an important practical result as regards the consistency of the depot fat itself. The "melting-points" of the fats and the setting-points of their mixed fatty acids (Table 1) are little, if at all, higher than those of the more usual ox depot fats with acids which include 40 % or more of oleic acid and the customary 30 % of palmitic acid; on the other hand, a fat with 30 % or less of oleic acid, 30 % palmitic acid and the balance in the form of stearic acid would possess a considerably higher melting-point. The increase in the proportion of palmitic, instead of stearic, acid is undoubtedly the reason why these Indian ox depot fats melt at temperatures little different from those of European, American or Australian tallows which contain considerably more combined oleic acid. At the body temperature of the oxen, therefore, fats of either type will possess much the same consistency or degree of fluidity.

Component glycerides of the Bombay and Calicut cow fats

The fats of highest and lowest palmitic acid content were selected for study of their glyceride structure. The fats were each resolved by systematic crystallization from acetone into three fractions of differing consistency and I.V., and each fraction was then studied by the methods of Hilditch & Paul [1938]; in the present paper only the numerical data at each stage of the analysis of both fats need be recorded.

Bombay cow depot fat

The fat (901 g.) was resolved into three fractions (A, B, C) by crystallization from acetone at 0° and 20°; the analytical characteristics and component acid percentages for each fraction are collected in Table 3.

Fully saturated glycerides in fractions A and B. These were isolated quantitatively after oxidation of each fraction in acetone solution with powdered $KMnO_4$ in the usual way.

Fraction A. The fat (100·7 g.) yielded 73·7 % (wt.) or 74·3 % (mol.)[1] of fully saturated glycerides which were further separated into three sub-fractions (D,

[1] The component acid figures for fraction A (Table 1) indicate that the minimum proportion of fully saturated glycerides present (i.e. with all unsaturated acids as mono-unsaturated-disaturated glycerides) must be 74·5 %; this figure has therefore been used.

Table 3. *Fractions from acetone (Bombay cow depot fat)*

	A		B		C	
Weight (g.)	391·8		254·4		254·8	
I.V.	7·9		30·1		51·1	
Sap. equiv.	280·3		282·1		284·6	
Glycerides % (wt.)	43·5		28·2		28·3	
Glycerides % (mol.)	43·8		28·2		28·0	
Component acids:	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Lauric	—	—	—	—	0·3	0·4
Myristic	6·1	7·1	3·8	4·4	4·5	5·4
Palmitic	42·8	44·7	39·9	41·9	32·0	33·6
Stearic	40·6	38·2	22·9	21·7	10·1	9·6
Arachidic	1·8	1·5	—	—	—	—
Tetradecenoic	Trace	0·1	0·2	0·3	0·9	1·0
Hexadecenoic	1·7	1·8	1·9	2·0	3·4	3·6
Oleic	7·0	6·6	30·0	28·6	44·6	42·5
Octadecadienoic	—	—	0·9	0·8	3·4	3·3
C_{20-22} unsaturated	—	—	0·4	0·3	0·8	0·6

E, F) by crystallization from ether. After component acid determinations on each sub-fraction, the glycerides present in each were calculated as binary mixtures of tripalmitin and dipalmitostearin, or of the latter and palmitodistearin,[1] with the results given in Table 4.

Table 4. *Components of fully saturated glycerides in fraction A (Bombay cow depot fat)*

Sub-fraction:	D	E	F	Total
Weight (g.)	35·9	25·5	12·8	74·2
Sap. equiv.	284·5	272·4	271·8	
Glycerides % (wt.)	48·5	34·4	17·1	
Glycerides % (mol.)	47·3	35·0	17·7	
Component acids:	% (mol.)	% (mol.)	% (mol.)	% (mol.)
Lauric	—	1·6	0·6	0·7
Myristic	1·0	13·5	22·2	9·1
Palmitic	43·5	55·7	45·5	48·1
Stearic	54·5	29·2	31·7	41·6
Arachidic	1·0	—	—	0·5
Component acid groups (increments % mol.):				
C_{16} ($+C_{14}+C_{12}$)	21·0	24·8	12·1	57·9
C_{18} ($+C_{20}$)	26·3	10·2	5·6	42·1
Probable component glycerides (increments % mol.):				
*Tri-"palmitin"	—	4·4	0·9	5·3
*Di-"palmito"-stearin	15·8	30·6	16·8	63·2
Palmitodistearin	31·5	—	—	31·5

* These will include, respectively, minor amounts of myristodipalmitin and myristopalmitostearins.

Fraction B. The fat (100·4 g.) yielded 11·0% (wt.) or 11·6% (mol.) of fully saturated glycerides, the component acids of which were lauric 1·1, myristic 32·3, palmitic 46·1 and stearic 20·5% (mol.). Its probable component glycerides were therefore tri-"palmitin" 39, di-"palmito"-stearin 61% (mol.).

[1] As usual, minor amounts of C_{14} and C_{12} acids are grouped, for component glyceride estimation, with palmitic acid, whilst stearic acid here includes minor amounts of arachidic acid and "oleic" acid those of the other minor component unsaturated acids.

Whilst the component acid data for the remaining mixed saturated-unsaturated glycerides of fractions A and B indicated that little or no tri-C_{18} glycerides were present therein, it was desirable to determine the proportion of tri-C_{18} glycerides, if any, in fraction C.

Tri-C_{18} glycerides in fraction C. The fat (120 g.) was hydrogenated as completely as possible (to I.V. 1·2) in presence of nickel at 170°, and the product crystallized exhaustively from dry ether at room temperature. The least soluble of the four sub-fractions obtained (42·7 % (wt.) or 42·4 % (mol.) of fraction C) contained as component acids myristic 1·5, palmitic 29·6, stearic 68·3, arachidic 0·5 and oleic 0·1 % (mol.); this corresponds with a minimum content of 6·8 % tri-C_{18} glycerides in this sub-fraction, or 2·9 % in the whole fraction C. (The three more soluble fractions included respectively 44·7, 42·6 and 45·8 % (mol.) of stearic acid in their component acids, suggesting the absence of tristearin.)

Table 5. *Probable component glycerides of the Bombay cow depot fat*

Fractions from acetone:	A		B		C	Total
Glycerides % (mol.)	43·8		28·2		28·0	100·0
Component acids (increments % mol.):						
Lauric	—		—		0·1	0·1
Myristic	3·1		1·2		1·5	5·8
Palmitic	19·6		11·8		9·4	40·8
Stearic	16·7		6·1		2·7	25·5
Arachidic	0·7		—		—	0·7
Tetradecenoic	Trace		0·1		0·3	0·4
Hexadecenoic	0·8		0·6		1·0	2·4
Oleic	2·9		8·1		11·9	22·9
Octadecadienoic	—		0·2		0·9	1·1
C_{20-22} unsaturated	—		0·1		0·2	0·3
	F.S.G. (32·6 %)	Mixed (11·2 %)	F.S.G. (3·3 %)	Mixed (24·9 %)	Mixed (28·0 %)	
Component acid groups (increments % mol.):						
C_{16} ($+C_{14}$, C_{12})	18·9	4·6	2·6	11·1	12·3	49·5
Stearic	13·7	3·7	0·7	5·4	2·7	26·2
Oleic ($+C_{20-22}$)	—	2·9	—	8·4	13·0	24·3
Component glyceride groups (increments % mol.):						
(*a*) Tripalmitin	1·7	—	1·3	—	—	3·0
Dipalmitomono-C_{18}	20·6	2·6	2·0	8·3	9·7	43·2
Palmitodi-C_{18}	10·3	8·6	—	16·6	17·5	53·0
Tri-C_{18}	—	—	—	—	0·8	0·8
(*b*) Fully saturated	32·6	—	3·3	—	—	35·9
Hexadeceno-disaturated	—	2·4	—	—	—	2·4
Mono-"oleo"-disaturated	—	8·8	—	24·6	17·8–17·0	51·2–50·4
Di-"oleo"-monosaturated	—	—	—	0·3	9·4–11·0	9·7–11·3
Tri-"olein"	—	—	—	—	0·8–Nil	0·8–Nil
Probable component glycerides (increments % mol.):						
Fully-saturated (35·9 %):						
Tripalmitin	1·7	—	1·3	—	—	3·0
Dipalmitostearin	20·6	—	2·0	—	—	22·6
Palmitodistearin	10·3	—	—	—	—	10·3
Mono-"oleo"-disaturated (53·6–52·8 %):						
Hexadecenopalmitostearin	—	2·4	—	—	—	2·4
"Oleo"-dipalmitin	—	0·2	—	8·3	9·7	18·2
"Oleo"-palmitostearin	—	8·6	—	16·3	8·1–7·3	33·0–32·2
Di-"oleo"-monosaturated (9·7–11·3 %):						
Palmitodi-"olein"	—	—	—	0·3	9·4–10·2	9·7–10·5
Stearodi-"olein"	—	—	—	—	Nil–0·8	Nil–0·8
Tri-"olein" (0·8–Nil %)	—	—	—	—	0·8–Nil	0·8–Nil

From the foregoing data the probable component glycerides of the whole Bombay cow depot fat are deduced to be those shown in the final column (bottom) of Table 5.

Calicut cow depot fat

The fat (904 g.) was resolved into three fractions (A, B, C) by crystallization from acetone at 0° and 20°; the analytical characteristics and component acid percentages for each fraction are collected in Table 6.

Table 6. *Fractions from acetone (Calicut cow depot fat)*

	A		B		C	
Weight (g.)	372·9		271·3		259·8	
I.V.	11·6		34·9		54·0	
Sap. equiv.	282·6		283·6		285·3	
Glycerides % (wt.)	41·3		30·0		28·7	
Glycerides % (mol.)	41·4		30·0		28·6	
Component acids:	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Lauric	—	—	Trace	Trace	1·3	1·7
Myristic	6·4	7·6	3·0	3·6	2·7	3·2
Palmitic	33·5	35·3	34·7	36·6	25·7	27·2
Stearic	45·1	42·8	23·3	22·2	12·7	12·2
Arachidic	1·5	1·3	—	—	—	—
Tetradecenoic	0·1	0·1	0·3	0·4	0·8	1·0
Hexadecenoic	0·8	0·9	1·3	1·4	2·4	2·5
Oleic	12·6	12·0	35·9	34·4	49·9	47·9
Octadecadienoic	—	—	1·5	1·4	4·1	4·0
C_{20-22} unsaturated	—	—	—	—	0·4	0·3

Fully saturated glycerides in fraction A. The fat (101 g.) yielded on oxidation in the usual manner 63·1 % (wt.) or 63·7 % (mol.) of fully saturated glycerides which were further separated into two sub-fractions (D, E) by crystallization from ether. The component acids present in each sub-fraction were determined; their molar percentages and the deduced binary mixtures of palmitostearins are given in Table 7.

Table 7. *Components of fully saturated glycerides in fraction A (Calicut cow depot fat)*

Sub-fraction:	D	E	Total
Weight (g.)	38·8	24·9	63·7
Sap. equiv.	283·1	274·6	
Glycerides % (wt.)	60·9	39·1	
Glycerides % (mol.)	60·2	39·8	
Component acids:	% (mol.)	% (mol.)	% (mol.)
Lauric	—	3·7	1·5
Myristic	4·1	15·5	8·6
Palmitic	41·3	42·9	42·0
Stearic	54·6	34·7	46·6
Arachidic	—	3·2	1·3
Component acid groups (increments % mol.):			
C_{16} (+C_{14}+C_{12})	27·4	24·7	52·1
C_{18} (+C_{20})	32·8	15·1	47·9
Probable component glycerides (increments % mol.):			
Di-"palmito"-stearin*	21·9	34·3	56·2
Palmitodistearin	38·3	5·5	43·8

* This will include minor amounts of myristo-(lauro-)palmitostearins.

Fully saturated glycerides in fraction B. The fat (119·6 g.) yielded 6·0 % (wt.) or 6·3 % (mol.) of fully saturated glycerides, of which the component acids were

myristic 35·9, palmitic 27·9 and stearic 36·2% (mol.). Its probable component glycerides were about 90% myristopalmitostearins (included in Table 8 with di-"palmito"-stearin) and 10% palmitodistearin.

Tri-C_{18} glycerides in fraction C. The fat (120 g.) was hydrogenated to I.V. 1·4 and separated into three fractions of increasing solubility in dry ether at room temperature. The least soluble of these fractions (41·0% (wt.) or 40·4% (mol.) of fraction C) contained as component acids palmitic 24·5, stearic 75·0, arachidic 0·4 and oleic 0·1% (mol.); this corresponds with a minimum content of 26·5% tri-C_{18} glycerides in this sub-fraction, or 10·7% in the whole fraction C. (The two more soluble fractions included respectively 56·5 and 51·2% (mol.) of stearic acid in their component acids, indicating substantial absence of tristearin.)

From the above data the probable component glycerides of the whole Calicut cow depot fat were deduced with the results shown in Table 8.

Table 8. *Probable component glycerides of the Calicut cow depot fat*

Fractions from acetone:	A		B		C	Total
Glycerides % (mol.)	41·4		30·0		28·6	100·0
Component acids (increments % mol.):						
Lauric	—		Trace		0·5	0·5
Myristic	3·2		1·1		0·9	5·2
Palmitic	14·6		11·0		7·8	33·4
Stearic	17·7		6·7		3·5	27·9
Arachidic	0·5		—		—	0·5
Tetradecenoic	Trace		0·1		0·3	0·4
Hexadecenoic	0·4		0·4		0·7	1·5
Oleic	5·0		10·3		13·7	29·0
Octadecadienoic	—		0·4		1·1	1·5
C_{20-22} unsaturated	—		—		0·1	0·1
	F.S.G. (26·4%)	Mixed (15·0%)	F.S.G. (1·9%)	Mixed (28·1%)	Mixed (28·6%)	
Component acid groups (increments % mol.):						
C_{16} (+C_{14}, C_{12})	13·8	4·4	1·2	11·4	10·2	41·0
Stearic	12·6	5·6	0·7	6·0	3·5	28·4
Oleic (+C_{20-22})	—	5·0	—	10·7	14·9	30·6
Component glyceride groups (increments % mol.):						
(*a*) Dipalmitomono-C_{18}	14·8	—	1·7	6·0	5·0	27·5
Palmitodi-C_{18}	11·6	13·2	0·2	22·1	20·5	67·6
Tri-C_{18}	—	1·8	—	—	3·1	4·9
(*b*) Fully saturated	26·4	—	1·9	—	—	28·3
Hexadeceno-disaturated	—	0·1	—	—	—	0·1
Mono-"oleo"-disaturated	—	14·9	—	23·9	15·5–12·4	54·3–51·2
Di-"oleo"-monosaturated	—	—	—	4·2	10·0–16·2	14·2–20·4
Tri-"olein"	—	—	—	—	3·1–Nil	3·1–Nil
Probable component glycerides (increments % mol.):						
Fully saturated (28·3%):						
Dipalmitostearin	14·8	—	1·7	—	—	16·5
Palmitodistearin	11·6	—	0·2	—	—	11·8
Mono-"oleo"-disaturated (54·5–51·3%):						
Hexadecenodistearin	—	0·1	—	—	—	0·1
"Oleo"-dipalmitin	—	—	—	6·0	5·0	11·0
"Oleo"-palmitostearin	—	13·1	—	17·9	10·5–7·4	41·5–38·4
"Oleo"-distearin	—	1·8	—	—	—	1·8
Di-"oleo"-monosaturated (14·2–20·4%):						
Palmitodi-"olein"	—	—	—	4·2	10·0–13·1	14·2–17·3
Stearodi-"olein"	—	—	—	—	Nil–3·1	Nil–3·1
Tri-"olein" (3·1–Nil %)	—	—	—	—	3·1–Nil	3·1–N

Comparison of the probable component glycerides of the depot fats from Bombay, Calicut and English oxen

It is interesting to compare the composition of the Bombay cow depot fat (unusually rich in combined palmitic acid) with that of the Calicut fat, which resembles the English ox depot fat [Hilditch & Paul, 1938] much more closely as regards its component fatty acids. The data for the component acids and the component glycerides (approximated to the nearest unit per cent) of these three ox depot fats are collected in Table 9. It is desirable again to point out that the methods involved in the present studies of component glycerides essentially restrict comparison to the three major component acids, palmitic, stearic and oleic, and that minor component acids have to be grouped with one or other of these three. Consequently the "oleo"-glycerides include the small amounts of octadecadienoic and C_{20-22} unsaturated acids, and stearo-glycerides also include traces of arachidic acid; whilst the "palmito"-glycerides take in, in addition to palmitic acid, the minor amounts of myristic (and lauric) and of hexa- (and tetra-) decenoic acids (apart from the least soluble portions of the Indian fats, where definite evidence of small amounts of hexadeceno-disaturated glycerides resulted). The circumstance that myristic acid amounts to 4–5% of the total component acids in ox depot fats involves the probable presence of about 12–15% of monomyristo-glycerides; these appear in Tables 6, 8 and 9 in the general category of di-"palmito"-mono-C_{18} glycerides, for the reason just mentioned.

Table 9. *Component acids (% mol.) and probable component glycerides (% mol.) of depot fats of Bombay, Calicut and English oxen*

	Bombay (cow) (present work)	Calicut (cow) (present work)	English (ox) (Hilditch & Paul [1938])
Component fatty acids:			
Lauric	0·1	0·5	0·2
Myristic	5·8	5·2	2·4
Palmitic	40·8	33·4	33·4
Stearic	25·5	27·9	21·4
Arachidic	0·7	0·5	1·3
Tetradecenoic	0·4*	0·4	0·6
Hexadecenoic	2·4	1·5	1·9
Oleic	22·9	29·0	35·2
Octadecadienoic	1·1	1·5	3·5
C_{20-22} unsaturated	0·3	0·1	0·1
Probable component glycerides:			
Fully saturated:			
Tripalmitin	3	—	3
Dipalmitostearin	23	16	8
Palmitodistearin	10	12	6
Mono-"oleo"-disaturated:			
Hexadecenopalmitostearin	2	Trace	—
"Oleo"-dipalmitin	18	11	15
"Oleo"-palmitostearin	33–32	41–38	35–32
"Oleo"-distearin	—	2	2
Di-"oleo"-monosaturated:			
Palmitodi-"olein"	10–11	14–17	20–23
Stearodi-"olein"	0–1	0–3	8–11
Tri-"oleins"	1–0	3–0	3–0

The glycerides in the Indian ox depot fats are of the mixed type characteristic of all other animal depot fats which have been examined, but it is instructive

to contrast the essential differences between the Bombay and Calicut specimens. The Calicut fat closely resembles the English fat in its palmitic acid content, the lower unsaturation of the Calicut fat being almost wholly accounted for by an increase of about 6% in stearic acid and a decrease of the same order in oleic acid as compared with the English fat. Concurrently, the glyceride structure is that hitherto regarded by us as typical for stearic-rich depot fats, and is fully explicable on the hypothesis of a biohydrogenation of preformed palmito-oleo-glycerides. Thus, the total proportions of monopalmitodi-C_{18} glycerides are 67% (Calicut) and 61% (English), but the more saturated Calicut fat contains about 6% more of both palmitodistearin and oleopalmitostearin than the English fat, whilst palmitodioleins are about 6% less in the Calicut than the English fat. Similarly the relative proportions of dipalmitostearin and oleodipalmitin are reversed in the two fats. The total amount of fully saturated components in the Calicut fat (28%) is consequently correspondingly larger than that in the English fat (17%).

The glyceride structure of the Bombay cow depot fat, however, marks a complete contrast to those of either of the other two fats. Here, as has already been indicated, stearic acid does not exceed the proportion in which it occurs in the Calicut fat, and the further fall in total unsaturation is compensated for by increase in the palmitic, and not the stearic, acid content. Whilst, therefore, the very low total unsaturation results in the presence of the highest proportion of fully saturated glycerides (36%) yet observed in an animal depot fat, these are for the most part dipalmito-glycerides. In the whole fat, the amount of monopalmitodi-C_{18} glycerides is only 53%, instead of approaching 70% as in other, more normal, ox depot fats hitherto examined [Banks & Hilditch, 1931; Hilditch & Paul, 1938]; dipalmitomono-C_{18} glycerides correspondingly exceed 40% compared with about 25% in the more usual types. (As already mentioned, the increase in dipalmitostearin and oleodipalmitin, instead of any further increase in palmitodistearin, has the effect of retaining the consistency or melting-point of this fat at about the same level as that of the Calicut or English ox depot fats which contain much more oleic acid.)

So far as the relative proportions of oleic and stearic groups in the various mixed glycerides are concerned, the Bombay cow fat exhibits the normal characteristics of a mixture of glycerides resulting from partial or complete hydrogenation of a mixture of preformed oleo-glycerides. Thus the proportion of fully saturated glycerides has the relationship to the proportion of saturated acids in the mixed acids of the fat which is in accordance with this hypothesis [Hilditch & Stainsby, 1935]; of the monopalmitodi-C_{18} glycerides, 21% are di-unsaturated, 60% mono-unsaturated and 19% palmitodistearin; whilst the dipalmitomono-C_{18} glycerides consist of 44% oleodipalmitin and 56% dipalmito-stearin. Comparison with the more unsaturated ox depot fat studied by Hilditch & Paul [1938, p. 1783] shows that the proportions of the more saturated groups of mono- and di-palmito-glycerides in the Bombay fat are increased, as would be expected on the hydrogenation hypothesis, and in spite of the unusually high proportions of palmitic acid (and, therefore, dipalmito-glycerides) present. It may be recalled that minor but progressive changes in the proportion of dipalmito-glycerides in depot fats have recently [Hilditch & Pedelty, 1940] been illustrated by comparison of pig and ox depot fats, in which a tendency for the more saturated (stearic-rich) fats also to contain slightly increased proportions of palmitic acid was observable. In the present instance, however, the increase in palmitic acid content of the Bombay cow depot fat compared with other animal depot fats is far more marked.

The recent studies of Schoenheimer and his colleagues appear very suggestive in this connexion. Schoenheimer & Rittenberg [1937] observed that the body fat of mice, fed for 5 days with deuterostearic acid, contained deuteropalmitic acid; and, conversely, Stetten & Schoenheimer [1940], after feeding ethyl deuteropalmitate to rats for 8 days, found that the body fats contained deuterostearic acid, deutero-acids of shorter chain length than palmitic acid and deuterohexadecenoic acid. These workers conclude that interconversions of fatty acids which take place normally and continuously in the animal body include not only those due to hydrogenation or dehydrogenation, but also those in which the carbon chain may be increased or diminished by two carbon atoms at a time. Schoenheimer's conclusions, if applicable to the glycerides and not merely to the fatty acids comprised in animal fats, offer a possible explanation of the present instance in which the usual oleic-stearic balance in a depot fat appears to have been superseded, beyond a certain limit in stearic acid content, by an oleic-palmitic or stearic-palmitic interconversion. It is further possible that Stetten & Schoenheimer's observations on the degradation of palmitic (and hence of stearic or oleic) groups to acids of shorter chain length have a similar bearing on the characteristic structure of cow milk fat, which has been similarly co-related with a mixture of preformed palmito-oleo-glycerides as a result of studies of its component glycerides [Hilditch, 1937; Hilditch & Paul, 1940].

Somewhat similar inter-relationships between the amounts of different saturated acids have been observed occasionally in a few fats of marine animals. Lovern [1932] found that in three different depot fats of the sturgeon the total molar percentage of saturated acids was approximately constant (24 %), but that it was made up of variable amounts of myristic, palmitic and stearic acids, whilst palmitic and hexadecenoic acids, individually varying by several units per cent., amounted together to a constant proportion of 40–41 % of the total fatty acids. Again, in the fats from five depots of the tunny, Lovern [1936] observed progressive relationships between the total content of saturated acids, the proportion of stearic acid, and the average unsaturation of the unsaturated C_{18} acids.

In conclusion, a further reference may be made to the proportions of "oleo"-dipalmitin, "oleo"-palmitostearin, palmitodi-"olein" and stearodi-"olein" present in the Bombay and Calicut cow depot fats. If, after allowing for the composition of the fully saturated glycerides present in the fats, the "oleic" acid present is divided proportionately between the palmitic and stearic acids present as mixed "oleo"-saturated glycerides, and combined arithmetically with these two acids, a "calculated" composition for the four mixed glycerides referred to is derived which represents the maximum "even distribution" of these acids in the form of mixed glycerides. In the case of the English ox depot fat [Hilditch & Paul, 1938], the figures obtained by calculation agreed closely with those actually observed, but this was not so in two pig depot fats subsequently examined [Hilditch & Pedelty, 1940]. The corresponding data for the two Indian cow depot fats, however, again display considerable accordance between the observed and "calculated" proportions of the respective glycerides. This similarity, the implication of which is uncertain, is shown by the appended figures:

	Bombay cow		Calicut cow		English ox	
	Found	"Calc."	Found	"Calc."	Found	"Calc."
"Oleo"-dipalmitin	18	23	11	13	15	14
"Oleo"-palmitostearin	32	33	38	39	32	35
Palmitodi-"olein"	11	6	17	13	23	22
Stearodi-"olein"	1	3	3	7	11	12

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SUMMARY

The component acids of four depot fats from oxen grown in various parts of India have been determined. All the fats were much more saturated than those of European or American animals, and contained only 27–33 % of unsaturated acids (mainly oleic). The stearic acid content of the fats did not exceed 26–28 %, however, and consequently the proportion of palmitic acid was in general considerably above the 30 ± 3 % hitherto considered characteristic for ox depot fats; in one instance (Bombay cow fat) palmitic acid formed over 40 % of the total fatty acids. The melting-points of the fats and the setting-points of their mixed fatty acids were of the same order as those of the more common and more unsaturated ox depot fats, owing to the presence of the increased proportions of palmitic acid.

The component glycerides of two of the cow depot fats have been studied in detail, and were found to conform with the general structure observed in other depot fats of oxen. In the Bombay cow fat the abnormal amount of palmitic acid was reflected in increased amounts of dipalmitostearin and oleodipalmitin but, apart from this, the general proportions of palmitodistearin, oleopalmitostearin, palmitodiolein etc. were in line with those in other ox depot fats, when allowance is made for the more highly saturated nature of the fat as a whole.

The apparent replacement of stearic acid, beyond a certain point, by palmitic acid is considered, with special reference to recent observations by Schoenheimer and to somewhat similar instances in certain marine animal fats.

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155. THE DISTRIBUTION AND CONCENTRATION OF ASCORBIC ACID IN THE POTATO (*SOLANUM TUBEROSUM*)

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(*Received 29 July 1940*)

SINCE the development of simple methods of estimating ascorbic acid, most fruits and vegetables have been assayed; a summary of the results has recently been prepared by Fixsen & Roscoe [1940]. Giroud [1938] reports a positive correlation in plant tissues between the ascorbic acid and the carotenoids and chlorophylls; Hausen [1935] states that the growth of plants is increased by the addition of ascorbic acid to the culture solution, whilst Virtanen [1936] finds that the concentration of the vitamin reaches a maximum during the early stages of growth. McHenry & Graham [1935] have found that ascorbic acid may be present in the reversibly oxidized form.

The values for potato tubers given by various investigators vary between about 5 and 40 mg. per 100 g. of tissue. These rather wide limits, as suggested in a previous paper by Smith & Paterson [1937], are partly accounted for by such factors as variety, health and storage conditions, so that the results obtained by different workers are not directly comparable. Normally stocks of potatoes to be sold for seed purposes must reach a certain standard of purity and freedom from certain virus diseases and for this purpose growing crops are graded by inspection. This means that during the dormant season the presence of virus in the tuber can be shown only by grafting experiments which take a long time to complete. The object of the present investigation was to make a comprehensive examination of the ascorbic acid content of the potato to see whether the incidence of virus infection were related to ascorbic acid content as had been indicated in earlier studies.

Methods

The indophenol method. Many methods for the determination of ascorbic acid have been described in recent years and, while most of them are based upon the reduction of 2:6-dichlorophenolindophenol, some workers make use of ascorbic acid oxidase known to be present in most plants. For the present investigation the indophenol method was adopted on account of its suitability for rapid routine determinations. It may be mentioned that indicators bearing the same name from two sources have been found to have different constitutions; the sodium salts had molecular weights of 290 and 340 so that the corresponding concentrations of 0·0005 *M* solutions were 0·0145 and 0·017 % respectively. The indicator was standardized by the method introduced by Menaker & Guerrant [1938]. This method compared well with the original method of Harris & Ray [1933] and had the advantage of being more easily carried out.

Tuber tissue. The methods of extraction depended upon the purpose of the experiment and the material examined. For example, to obtain representative

samples of tuber tissue, cylinders were cut in the direction of the major axis with a cork borer and portions of them were cut off with parallel knives, each portion weighing about 2 g. For the purpose of obtaining an idea of the concentration of ascorbic acid these pieces, which were always of the same dimensions, were simply shaken up with 10 ml. of 2% trichloroacetic acid for 1 min. and the supernatant liquid was titrated with standard indophenol. The results so obtained are referred to as "indophenol values". For complete extraction, the cores were immediately ground to a pulp with about 25 ml. of trichloroacetic acid and about 8 g. of pure sand. The extraction was repeated three times, the extract being filtered through glass wool into a standard flask and made up to a definite volume. It was sometimes necessary to centrifuge the samples and titrate an aliquot portion of the clear liquid.

Sprouts and leaves. The method used for sprouts and leaves was the same as for tuber cores but the amounts of material were much smaller and a microburette was used for the titrations. Many of these extracts were deeply coloured and had to be specially dealt with. The method used was based upon that introduced by McHenry & Graham [1935] which depends upon the fact that the plant pigments are insoluble in $CHCl_3$. The technique was essentially that of Olliver [1938], the ascorbic acid solution being placed in a narrow tube with 2 ml. of $CHCl_3$. Stirring was carried out by a stream of N_2 and the indicator was added until the $CHCl_3$ layer developed a definite pink colour. It was found that thorough stirring of the extract and the indicator was advisable before mixing the $CHCl_3$ and aqueous layers and sometimes it was necessary to centrifuge in order to break the emulsion.

Dehydroascorbic acid. A certain amount of dehydroascorbic acid was almost always found in potato tubers, although the amount varied considerably during the season. The total ascorbic acid was determined by passing H_2S for 15 min. into the extract (frothing could be reduced by the addition of 2 ml. of *iso*propyl alcohol) and allowing it to stand overnight in a stoppered flask. The excess of H_2S was then driven off with a stream of oxygen-free N_2 before carrying out the indophenol titration. The dehydroascorbic acid was calculated by subtracting from this total value the amount of ascorbic acid obtained by the usual method. There seems to be no reason to suspect that other reducing substances are formed in tuber extracts by this treatment; little or no dehydroascorbic acid could be found, for example, in tubers examined immediately after harvesting.

Freezing. The above processes are rather tedious for the examination of large numbers of samples and an attempt was made to save time by freezing the samples. Solid CO_2 was first used, the cores being packed for a few minutes into holes cut in a solid block of CO_2; this offered no advantage over the usual method because the frozen cores had subsequently to be ground up. Liquid air was also tried, the cores being placed in a small muslin bag or test tube, immersed in the liquid and then ground. A serious loss of ascorbic acid, however, always resulted from the freezing, the loss not being entirely accounted for by the formation of dehydroascorbic acid. For example, the average amount of ascorbic acid for 4 varieties was 7·5 mg. per 100 g. of unfrozen tissue and only 1·8 mg. per 100 g. of material after freezing; the amount of reversibly oxidized ascorbic acid accounted for only about half of the difference. Mills [1932] found similar results when liver was frozen. It is perhaps worth noting that a pure solution of ascorbic acid in 2% trichloroacetic acid did not show this loss of ascorbic acid on freezing except in presence of tuber tissue.

Results

Diurnal fluctuations in leaves. Preliminary tests showed that the hour of sampling had an important effect on the ascorbic acid content of the leaves and stem. A few plants of each variety were marked and leaflets were taken at intervals and immediately placed in trichloroacetic acid. The determination of the ascorbic acid was carried out as already described. The two varieties used were Kerr's Pink and Epicure and samples were taken in the various experiments at intervals of up to 48 hr. The tests were conducted about the middle of June when the tubers were beginning to form and the plants were about 18 in. high. Figs. 1 and 2 illustrate the nature of the results obtained and show that the

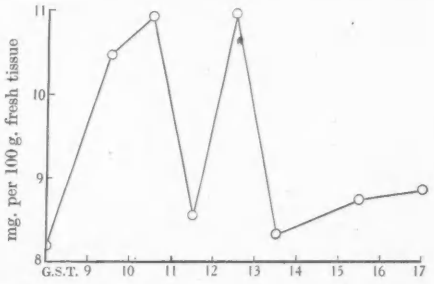

Fig. 1. Diurnal fluctuation of concentration of ascorbic acid in leaves of Kerr's Pink.

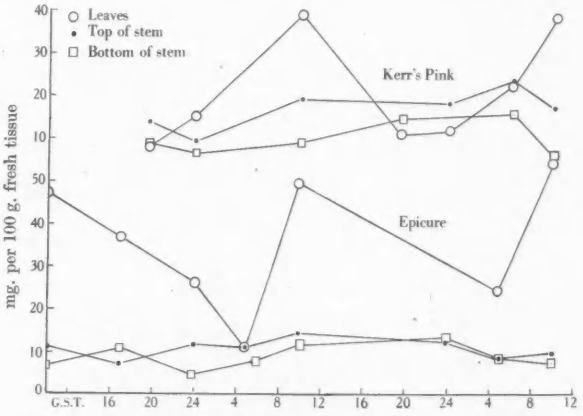

Fig. 2. Variation in concentration of ascorbic acid in leaves and stem of Kerr's Pink and Epicure with time.

fluctuations in the concentration of ascorbic acid are not unlike those for the nitrogen [Barton-Wright & McBain, 1933] and carbohydrate [Cockerham, 1939] in the plant. The concentration reaches a maximum early in the forenoon; the top of the plant has almost always a higher concentration than the lower part, the variations at the base of the stem being comparatively small.

Seasonal variations. Samples were always taken at 10 a.m. starting about the beginning of July and continuing until the end of the growing season. At one centre, both healthy tubers and tubers infected with severe mosaic and leaf roll belonging to 3 varieties (Arran Pilot, Arran Consul and Dunbar Cavalier) were planted and the leaflets nearest the growing tips of numbered plants were sampled along with tubers from the same plants. The figures showed that, on an average, the concentration of ascorbic acid in the leaves was twice as great as that in the tubers. The differences between healthy and diseased plants were rather irregular. In the second locality, 7 varieties were planted and the results are given in Table 1. It is obvious that the concentration of ascorbic acid in the young tuber varies with the stage of development, the maximum being reached some time in August, depending upon whether the variety is early, second early or late. There follows a sharp fall in concentration as the haulms ripen and die down. At all stages in the growth, the leaves have a much higher concentration than the tubers.

Table 1. *Ascorbic acid (mg./100 g.) in leaves (L) and tubers (T) at different times during the growing season*

Date ...	4 July		26 July		31 Aug.		14 Sept.	
Variety	L	T	L	T	L	T	L	T
Arran Pilot	120	27	101	14	59	30	—	17
Great Scot	84	17	76	25	54	26	—	18
Kerr's Pink	—	—	75	25	51	31	—	14
Arran Banner	—	—	90	20	60	34	—	22
Redskin	—	—	46	21	60	24	—	14
Majestic	—	—	49	21	52	30	—	14
Dunbar Cavalier	—	—	62	21	66	24	—	14

Effect of manuring. Ten plots were laid down in duplicate. The variety of potato chosen was Redskin of stock seed standard[1]. In addition to a basal dressing of farmyard manure, varying amounts of N, K and P were added in the form of ammonium sulphate, potassium sulphate or nitrate and superphosphate. The tubers were harvested at the end of September, stored at a temperature of 8–10° for a period of 3 weeks and the concentration of ascorbic acid was determined as previously described. The results varied between 14·4 and 16·7 mg. per 100 g. of tissue, but it was not possible to correlate the differences with manurial treatment. This agrees with the results previously found by Smith & Paterson [1937]. Wachholder & Nehring [1938] also reported that farmyard and artificial manures made no substantial differences to the ascorbic acid content of a number of varieties of potatoes, whilst Virtanen [1936] found that, although unbalanced manuring lowered the vitamin C content of potato tubers, any adequate treatment gave almost constant values.

The ascorbic acid in mature tubers

The effect of storage. It was impossible to examine all the material immediately after harvesting and consequently it was necessary to discover how the results might be affected by storage. From previous work there was reason to suspect that the ascorbic acid decreased considerably during storage and that the amount of dehydroascorbic acid was very small at the time of lifting. The latter conclusion was confirmed by determining the amount of dehydroascorbic acid in a series of tubers of each of 6 varieties immediately after lifting in September. It is

[1] This is the highest standard of purity and health as recognized by field inspection.

unnecessary to give all the figures for they were remarkably uniform for the different varieties; the average values for ascorbic and dehydroascorbic acids were respectively 16·5 and 0·3 mg. per 100 g. of tissue. Further determinations, however, showed that the amount of dehydroascorbic acid increased fairly rapidly during storage. The results are given in Table 2 which shows the ascorbic and dehydroascorbic acid contents after different periods of storage at temperatures varying between 2° and 8°. In every case there was a substantial decrease in the amount of ascorbic acid during storage, the values after storing for 6 months being only about one-third of those after 1 month's storage. Similar results have recently been reported by Zilva & Barker [1939] in connexion with one variety which they studied. The proportion of dehydroascorbic acid increased during storage and amounted, on the average, after 6 months to more than a third of the ascorbic acid. This increase in dehydroascorbic acid does not account for the observed loss of ascorbic acid. In fact the amount of dehydroascorbic acid tends to remain constant, which would seem to indicate that it is formed at the expense of ascorbic acid and then further oxidized to an irreversibly oxidized form.

Table 2. *Ascorbic (A) and dehydroascorbic acid (D) in mg. per 100 g. tuber tissue after different periods of storage*

Storage time ...	4 weeks		2 months		4 months		6 months	
Variety	A	D	A	D	A	D	A	D
Arran Consul	16·4	2·9	8·7	1·6	4·9	3·9	5·2	2·7
Arran Chief	—	—	10·8	1·2	5·7	2·3	6·4	2·1
Di-Vernon	—	—	8·8	1·6	6·2	1·3	7·5	3·8
President	—	—	—	—	6·7	—	5·6	2·7
Dunbar Cavalier	13·9	3·3	11·3	0·7	7·1	0·8	6·3	3·4
Majestic	15·4	2·9	8·6	1·4	7·4	1·5	5·5	1·7
Doon Star	—	—	12·7	4·1	7·6	0·9	5·3	4·2
Epicure	—	—	—	—	7·7	1·2	5·2	1·8
Dunbar Standard	—	—	—	—	7·7	1·2	7·5	2·8
King Edward	23·4	1·1	18·8	2·4	8·1	1·1	5·6	2·4
Duke of York	—	—	—	—	8·3	1·8	7·4	2·4
Up-to-Date	19·2	2·0	—	—	8·5	1·7	8·0	1·8
Arran Banner	—	—	8·6	3·9	8·7	1·1	7·8	2·2
Gladstone	—	—	—	—	9·3	1·4	6·4	1·2
Redskin	26·7	1·6	12·2	1·6	9·7	2·9	5·1	4·4
Golden Wonder	19·4	0·6	10·9	1·8	9·8	1·7	6·6	2·6
Great Scot	20·1	4·3	10·4	1·6	—	—	7·2	1·8
Kerr's Pink	22·4	2·0	12·9	3·0	—	—	9·5	1·5
Average	19·7	2·3	11·2	2·1	7·7	1·5	6·6	2·5

The effect of variety. After any particular period of storage there are differences associated with the varieties which in many cases are outside the range of experimental error. It would be dangerous to emphasize these differences too much on account of the very large part played by storage. It will be noticed, for example, that the order of varieties according to ascorbic acid concentration is not constant at the various periods of storage. The results given in Table 2 are average figures obtained from several tubers from one source so that the influence of locality is not shown. Tubers from 4 other sources were also examined but the analyses unfortunately extended over a period of about 6 weeks and the data are consequently not strictly comparable. There was, however, a marked tendency for the results from one source to be higher than those from the others, but it is not known whether this was due to stage of maturity when lifted or to conditions of storage. It is unlikely that the difference was due to climate because "stock

seed" is generally produced in the northern counties and, as already indicated, differences in normal manuring practice would not affect the results.

The effect of disease. One of the objects of the investigation was to examine the correlation, suggested by earlier work, between the amount of ascorbic acid in the tuber and the presence or absence of virus disease. The greatest difficulty in this type of work is the obtaining of tubers which can be guaranteed to be free from infection or to be infected with known viruses. For the most part we had to accept as healthy material tubers which had been placed in "stock seed" categories by field inspection of crops, whilst the infected material was taken from plants which were observed in the field to be suffering from severe mosaic or leaf roll. It has been shown [Scott, 1938] that "stock seed" tubers are not necessarily free from virus, and it is well known that mosaic may be due to a combination of viruses. The results obtained from the examination of many varieties, healthy and infected with severe mosaic or leaf roll, are summarized in Table 3. It will be noticed that after 1 month's storage 13 out of 17 varieties examined show a greater concentration of ascorbic acid in the infected than in the healthy tubers. After 4 months' storage, the results are not so striking and those for ascorbic and dehydroascorbic acid together are not more conclusive. Indophenol values were also obtained by the quick method of extraction previously described, and here it will be seen that most varieties again show a higher value for infected than for healthy material. The results for leaf roll material are not so complete but once again there is a tendency for the concentration of ascorbic acid to be greater in the diseased than in the healthy tubers.

Table 3. *Ascorbic (A) and dehydroascorbic acid (D) in mg. per 100 g. tissue and "indophenol value" (I) for healthy and diseased tubers after 1 and 4 months' storage*

Storage ...	1 month				4 months				1 month		
	A		D		A		D		Indophenol value		
Variety	H	M	H	M	H	M	H	M	H	M	L.R.
Arran Consul	16·4	16·8	2·9	4·2	4·9	5·3	3·9	1·8	0·46	0·54	—
Arran Chief	15·4	16·1	—	—	—	—	—	—	0·45	0·52	—
Di-Vernon	—	—	—	—	—	—	—	—	0·56	0·87	—
Dunbar Cavalier	13·9	26·4	3·3	0·1	7·1	7·5	0·8	0·5	0·65	0·61	—
Majestic	15·4	18·4	2·9	3·1	7·4	7·2	1·5	0·7	0·44	0·41	0·37
Doon Star	19·0	21·0	—	—	7·6	6·9	0·9	4·3	0·46	0·51	0·49
King Edward	23·4	28·3	1·1	0·0	8·1	9·3	1·1	5·4	0·96	1·21	1·62
Duke of York	24·0	22·0	—	—	8·3	11·7	1·8	1·2	—	—	—
Up-to-Date	19·2	20·6	2·0	4·2	8·5	9·9	1·7	0·5	0·90	1·04	0·91
Arran Banner	22·3	25·0	—	—	8·7	7·7	1·1	4·0	0·89	0·94	1·04
Gladstone	22·4	19·3	—	—	—	—	—	—	0·93	—	1·02
Redskin	26·7	24·7	1·6	1·2	9·7	10·1	2·9	2·0	0·63	0·91	0·80
Golden Wonder	19·4	17·5	0·6	0·0	9·8	5·7	1·7	4·1	0·60	0·60	0·67
Great Scot	20·1	21·3	4·3	1·1	8·5	8·9	—	—	0·55	0·55	—
Kerr's Pink	22·4	29·8	2·0	2·0	11·8	9·3	—	—	1·07	1·27	0·99
Arran Pilot	18·8	23·3	13·8	5·5	9·8	9·5	—	—	0·72	0·70	—
Catriona	24·9	31·6	—	—	—	—	—	—	1·32	1·27	—
Sharpe's Express	24·1	25·2	—	—	—	—	—	—	1·14	1·37	—
Eclipse	—	—	—	—	—	—	—	—	0·55	1·05	0·53

H = healthy; M = severe mosaic; L.R. = leaf roll.

Several factors have to be borne in mind when considering these results. Some doubt must always exist with respect to the freedom of the healthy material from infection. Further, there is the question of the time of storage,

for, although storage in the laboratory could be controlled, it was not possible to obtain precise information from various growers as to the date of lifting and the conditions of storage between lifting and dispatch to the laboratory. These results should not, therefore, be stressed unduly; nevertheless, the recurrence of the difference between healthy and diseased material would seem to be more than a mere accident. In order to make a proper study of this question it would be necessary to carry out a long term investigation with material specially selected and tested and grown under those conditions which are necessary for carrying out virus research work.

Distribution of ascorbic acid in the potato tuber

Various sections of the outer layer and of the internal tissue of the tuber were examined. The concentration of ascorbic acid on the surface varied rather indefinitely but it was greater at the rose than at the heel end; in the inner tissue it was also greater at the rose than at the heel end, and, in fact, showed a consistent gradation. Typical results for a core running from the heel to the rose end and divided into 6 sections are given in Table 4.

Table 4. *Distribution of ascorbic acid within tuber tissue*

	Heel		Centre		Rose	
Section ...	1	2	3	4	5	6
Ascorbic acid (mg./100 g.)	4·3	5·5	6·8	7·1	7·6	8·1

These results were obtained after sprouting had begun and do not necessarily represent the normal distribution in the tuber throughout the dormant season.

Ascorbic acid in tubers and sprouts grown in the light and the dark. For the purpose of this investigation 12 tubers of each of 4 varieties were placed on trays exposed to the light and comparable lots of 12 tubers were kept on trays in the dark. The varieties were Golden Wonder, King Edward, Great Scot and Arran Pilot. After sprouting was sufficiently advanced, 6 sections were cut from each sprouted tuber. In every case when the tuber had been exposed to the light there was an increase in the concentration of ascorbic acid from the heel end of the tuber to the middle or tip of the sprout; when the tuber had sprouted in the dark the concentration gradient was not so steep and not quite so regular and the highest concentration was, with one exception, found in the middle of the sprout. The concentration of ascorbic acid in all cases was greater in the middle and tip of those sprouts exposed to the light than in the corresponding positions of those sprouts which had developed in the dark. Table 5 contains the average values for the 4 varieties and gives a very good idea of the nature of the gradient in tubers growing in the light and in the dark.

Table 5. *Distribution of ascorbic acid (mg./100 g.) between tuber and sprout*

	Tuber			Sprout		
Position ...	Heel	Middle	Rose	Base	Middle	Tip
Light	6·2	6·7	8·8	10·0	14·5	14·3
Dark	7·7	7·1	10·1	11·1	12·5	8·8

Source of ascorbic acid in the growing plant. Several tubers of each of 4 varieties were weighed and placed in trays exposed to the light and a corresponding set was stored in the dark. The varieties were Great Scot, King Edward, Golden Wonder and Redskin. At the start of the experiment the average concentration

of ascorbic acid in the tubers of each of the 4 varieties was determined so that the total amount of ascorbic acid in each tuber set aside to sprout was known. In due course the sprouts of each tuber were cut off, the sprouts and tubers weighed separately and the ascorbic acid contents determined. From these data it was possible to calculate how much ascorbic acid had been lost from the tuber since the start of the experiment and to say whether or not ascorbic acid had been synthesized. The results for 4 tubers of each variety are given in Table 6. They include figures for dehydroascorbic acid in the case of the variety Redskin.

Table 6. *Transfer of ascorbic acid from tuber to sprout*

		Tubers							Sprouts			
	Date ...	14. ii. 40			6. v. 40			Loss of A from tuber	6. v. 40			Net loss of A
Storage	Variety	W	A	D	W	A	D		W	A	D	
Light	Great Scot	500	53·0	—	421	41·6	—	11·4	12·8	2·8	—	8·6
	King Edward	403	29·8	—	335	22·1	—	7·7	6·2	1·8	—	5·9
	Golden Wonder	372	36·5	—	282	25·8	—	10·7	6·7	1·4	—	9·3
	Redskin	469	51·6	13·1	376	40·5	6·7	11·1	8·7	1·8	1·2	9·3
Dark	Great Scot	554	58·7	—	386	37·0	—	21·7	68·0	4·6	—	17·1
	King Edward	366	27·1	—	303	23·9	—	3·2	12·5	1·8	—	1·4
	Golden Wonder	385	37·8	—	281	23·5	—	14·3	25·1	2·2	—	12·1
	Redskin	432	47·5	12·1	280	27·3	1·4	20·2	37·7	3·0	2·0	17·2

W = weight of tubers or sprouts in g.
A = weight of ascorbic acid in mg.
D = weight of dehydroascorbic acid in mg.

These results confirm the conclusion drawn from the previous section, that there is a greater concentration of ascorbic acid in the sprouts grown in the light than in those grown in the dark. The actual weight of ascorbic acid present in the sprouts grown in the dark is greater, however, due to the much greater weight of sprout tissue. Over the 3 months during which sprouting had been going on there was an average net loss of ascorbic acid of 1·9 and 2·8 mg. per 100 g. original tuber tissue when sprouted in the light and dark respectively. This indicated that ascorbic acid had not been synthesized to any extent at this stage in the life cycle, but was simply passing from the tuber to the sprout. There was also a net loss of dehydroascorbic acid which was greater in the case of storage in the dark. It is possible that the loss is connected with the increase in respiration due to sprout formation and this would account for the greater loss in the case of the long etiolated sprouts formed in the dark.

Summary

The concentration of ascorbic acid in the leaves fluctuates considerably during the day but shows a maximum value in the early forenoon; it is on the average much greater in the leaves than in the tubers. In the tubers it reaches a maximum in August and falls quickly as the plant ripens off. During storage the value decreases until, after 6 months, it is only about one-third of the value at the time of harvesting. Variations in normal manuring practice do not produce significant effects. There is usually a greater concentration in tubers from plants affected with severe mosaic or leaf roll than in tubers presumed to be healthy. The concentration of ascorbic acid increases from the heel to the middle or tip of the sprout; the gradient is more pronounced when sprouting has taken place in the light than when it has occurred in the dark. There is apparently no synthesis of ascorbic acid during the early stages of sprouting but rather a net loss which is greater when sprouting has taken place in the dark.

Part of this investigation was carried out with the aid of a grant from the Agricultural Research Council and the Department of Agriculture for Scotland for which the authors are grateful. They would also like to acknowledge their indebtedness to Dr Cockerham, Dr Robertson and Mr Scott for advice and assistance in securing special material and to many merchants and farmers for supplies of tubers for the investigation.

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156. THE EFFECT OF VITAMIN E DEFICIENCY ON THE VITAMIN A RESERVES OF THE RAT

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(*Received 22 July 1940*)

In early work in this laboratory on vitamin E deficiency in the rat a basal diet was used in which vitamin A was supplied as cod liver oil (*ca.* 1000 I.U. per g.). The oil was given at the rate of about 70 mg. per rat weekly. Since from the condition of some of the animals it was suspected that this level was not adequate, livers were taken at autopsy and the vitamin A in them was assayed by the $SbCl_3$ method. The vitamin A reserves were found to be absent or low. It is questionable whether at this comparatively low level of intake of vitamin A any other result was to be expected, but in subsequent experiments the vitamin A allowance was increased to 17 mg. weekly of halibut liver oil (*ca.* 60,000 I.U. per g.). Determinations of liver reserves were continued. The results of these determinations, which have been reported in preliminary papers [Moore *et al.* 1939; Moore, 1939], are now given in detail. They indicate that in deficiency of vitamin E the vitamin A reserves of the liver may be much reduced.

Opportunities arose in other experiments to compare the vitamin A reserves of rats having diets low in protein, with or without simultaneous deficiency of vitamin E. In contrast with the marked and consistent reduction of the vitamin A reserves in vitamin E deficiency the differences ascribable to protein deficiency were irregular and slight, and never passed beyond the range of difference in body weight found in the groups receiving normal and low protein diets.

The efficiency of the conversion of carotene into vitamin A in avitaminosis-E was also investigated and a slight reduction in the amount of vitamin A formed was found under the experimental conditions adopted.

Experimental

Rats were killed by coal gas after being kept for varying periods on a diet deficient in vitamin E (light white casein 25%, cane sugar 50%, lard 10%, salt mixture 5%, dried yeast 10%). The animals used as positive controls received orally either a concentrate of vitamin E from wheat germ oil supplied by Glaxo Laboratories, Ltd., or a solution in arachis oil of *dl*-α-tocopherol or its acetate kindly presented by Roche Products, Ltd. At autopsy the livers were digested with alkali, the oils extracted with ether and alcohol, and their vitamin A was determined by the $SbCl_3$ method [Davies, 1933]. To convert blue units into I.U. the approximate factor of 0·6 has been used [Moore, 1937].

Vitamin A allowance. Except where otherwise stated vitamin A was given orally by pipette as one drop (17 mg.) of halibut liver oil per rat per week. The oil was kindly supplied by Allen and Hanburys, Ltd., and contained 60,000 I.U. of vitamin A per g. The vitamin A intake as halibut liver oil was therefore about 1000 I.U. per week. In addition the rats given wheat germ oil concentrate probably received small amounts of carotene from this source. No attempt was made to

identify carotene in the concentrate, but a colorimetric determination of the total epiphasic pigments indicated a maximum carotene content of about 2000 I.U. per g., or 100 I.U. in the weekly allowance. Since the epiphasic pigments may have included artifacts and other pigments inactive as provitamins it seems probable that this estimate is unduly high.

The effect of wheat germ oil concentrate on vitamin A reserves (*Exp. 1, Table 1*). Groups of female albino rats were restricted to the diet deficient in vitamin E at a mean initial weight of 68 g. and were killed 56 weeks later. The deficient rats were by then in a state of advanced avitaminosis-E, with severe paralysis of the hind legs and deep brown uteri [Martin & Moore, 1936; 1938; 1939]. The positive control animals received 50 mg. of wheat germ oil concentrate daily and were in good health.

Table 1. *The storage of vitamin A by rats with and without vitamin E (wheat germ oil concentrate)*

Exp. 1

	− vitamin E				+ vitamin E			
			I.U. of vitamin A				I.U. of vitamin A	
	Wt. of rat g.	Wt. of liver g.	Total	Per g. liver	Wt. of rat g.	Wt. of liver g.	Total	Per g. liver
	126	6·0	6,000	1000	215	12·0	19,000	1600
	145	7·5	9,000	1200	210	9·0	26,000	2900
	150	10·8	15,000	1400	237	12·0	30,000	2500
	144	10·4	15,000	1400	223	13·5	30,000	2200
Means	141	8·7	11,300	1250	221	11·6	26,300	2300

The vitamin A reserves of the rats given vitamin E were invariably much higher than those of the rats deficient in vitamin E. The positive control group had a mean total vitamin A reserve more than twice as great as that of the deficient group. The reserve per g. of liver for the control group was almost twice as much as the corresponding value for the deficient group.

Exp. 2, Table 2. Rats were given the diet deficient in vitamin E at a mean weight of 37 g. and were killed 33 weeks later. Control animals received weekly doses of wheat germ oil concentrate. In parallel experiments similar groups of

Table 2. *The storage of vitamin A by rats with and without vitamin E (wheat germ oil concentrate) and with normal and reduced protein allowances*

Exp. 2

		− vitamin E				+ vitamin E			
				I.U. of vitamin A				I.U. of vitamin A	
		Wt. of rat g.	Wt. of liver g.	Total	Per g. liver	Wt. of rat g.	Wt. of liver g.	Total	Per g. liver
Normal casein		170	9·0	2000	130	179	9·2	12,000	1300
		163	10·6	1500	140	204	10·6	15,000	1400
		153	7·9	1500	190	185	9·8	15,000	1500
	Means	162	9·2	1400	153	189	9·9	14,000	1400
Low casein		95	5·5	600	110	112	9·1	12,000	1300
		66	4	600	150	110	6·6	12,000	1800
		80	5·2	750	140	(88)*	(3·1)*	(7,500)*	(2400)*
	Means	80	4·9	650	133	111	7·8	12,000	1550

* Died after 203 days; value excluded in taking mean.

rats received a modification of the usual basal diet in which the caseinogen was reduced to 3%, the difference in energy value being made up by additional sugar.

The findings at autopsy were as follows: *Normal casein+vitamin E*: uteri white, good intraperitoneal fat reserves. *Normal casein−vitamin E*: uteri brown, sparse fat reserves. *Low casein+vitamin E*: rats undersized, white uteri, sparse fat reserves. *Low casein−vitamin E*: rats undersized, small brown uteri, very sparse fat reserves.

The mean vitamin A reserves, both total and per g., were about 10 times greater in the groups given vitamin E concentrate than in the deficient groups. Casein deficiency had a relatively small effect of doubtful significance. The group given a normal allowance of casein without vitamin E had total reserves about twice as great as those given a low allowance of casein without vitamin E. When the reserves are calculated per g. of liver this difference disappears.

Exp. 3, Table 3. This was in principle a repetition of Exp. 2. The rats were kept on a slightly modified diet deficient in vitamin E and also low in vitamin A (light white casein 25%, rice starch 50%, lard 20%, salts 5%+dried yeast 15%) from lactation until they were about 10 weeks old and had a mean weight of 124 g. They were then divided into groups, transferred to the standard vitamin E-free diet and treated exactly as in the preceding experiment. The findings at autopsy after a further 32 weeks were as follows: *Normal casein +vitamin E*: large fat reserves, uteri white. *Normal casein−vitamin E*: sparse fat reserves, uteri brown. *Low casein+vitamin E*: sparse fat reserves, uteri white. *Low casein−vitamin E*: very sparse fat reserves, small brown uteri.

Table 3. *The storage of vitamin A by rats with and without vitamin E (wheat germ oil concentrate) and with normal and reduced protein allowances*

	Exp. 3							
	−vitamin E				+vitamin E			
			I.U. of vitamin A				I.U. of vitamin A	
	Wt. of rat g.	Wt. of liver g.	Total	Per g. liver	Wt. of rat g.	Wt. of liver g.	Total	Per g. liver
Normal casein	164	8·4	1200	140	249	12·5	15,000	1200
	164	10·0	1800	180	238	12·0	15,000	1300
	164	8·8	1800	200	264	13·2	18,000	1400
Means	164	9·1	1600	173	250	12·6	16,000	1300
Low casein	125	8·4	1200	140	156	9·6	7,500	780
	107	8·2	1500	180	149	9·0	7,500	840
	115	8·0	1500	190	132	8·0	9,000	1100
Means	116	8·2	1400	170	146	8·9	8,000	906

The vitamin A reserves were again much lower in the deficient groups than in those given vitamin E. The casein allowance had no effect on the vitamin A reserves in the groups deficient in vitamin E. In the groups given vitamin E the total reserves were about twice as great with a normal casein allowance as with a low allowance, but this difference disappeared when the results were calculated per g. of liver.

The effect of tocopherol on vitamin A reserves (Exp. 4, Table 4). The rats were given the modified diet deficient in vitamin E from weaning until attaining an age of about 8 weeks. One drop of halibut liver oil weekly was given during

this period. The rats were then transferred to the standard vitamin-E free diet, 6 groups of 2 animals each being formed. Four groups were given weekly doses of 3, 1, 0·3 and 0·1 mg. of tocopherol dissolved in arachis oil. The remaining groups received 50 mg. of arachis oil and 50 mg. of wheat germ oil concentrate respectively. For reasons of convenience some of the rats were killed 24 weeks and some 29 weeks after the commencement of the administration of tocopherol.

Table 4. *The effect of graded doses of* dl-α-*tocopherol on the storage of vitamin A by the rat*

Exp. 4

Weekly dose of vitamin E mg.	Duration of exp. weeks	Wt. of rat (initial and final) g.	Wt. of liver g.	Colour of uterus	Vitamin A reserve I.U. Total	Per g. liver
Tocopherol 3	24	106–198	10·8	White	12,000	1100
	24	129–219	10·2	White	15,000	1500
				Means	13,500	1300
Tocopherol 1	29	95–196	10·3	White	12,000	1200
	29	122–230	12·7	White	15,000	1200
				Means	13,500	1200
Tocopherol 0·3	29	109–229	10·4	Brown	6,700	650
	29	125–240	13·8	Brown	7,500	550
				Means	7,100	600
Tocopherol 0·1	29	113–198	8·7	Brown	3,700	430
	29	121–231	10·8	Brown	6,000	560
				Means	4,850	495
Nil	24	128–175	11·6	Brown	3,300	280
	24	112–169	11·8	Brown	6,000	510
				Means	4,650	395
Wheat germ oil concentrate 50	24	132–221	10·0	White	15,000	1500
	24	113–208	9·8	White	15,000	1500
				Means	15,000	1500

The uteri of rats in the groups given 3 and 1 mg. of tocopherol or 50 mg. of wheat germ oil concentrate weekly were normal in colour. The uteri of rats given 0·3 and 0·1 mg. of tocopherol or plain arachis oil were brown, and the vitamin A reserves for these groups were only $\frac{1}{4}$ to $\frac{1}{2}$ of those found in the groups receiving adequate allowances of vitamin E.

It may be noticed that the reserves of the rats given wheat germ oil concentrate were not significantly greater than those of the groups given 3 and 1 mg. of tocopherol. This affords evidence that the ability of wheat germ oil concentrate to promote the storage of vitamin A, as demonstrated in the present and preceding experiments, was due to the vitamin E which it contained and not to the presence in it of unsuspectedly large amounts of carotene, or to its contamination with vitamin A.

The effect of tocopherol on the conversion of carotene into vitamin A (*Exp. 5, Table 5*). The rats were kept on the modified vitamin E-deficient diet from weaning until attaining an age of about 16 weeks. Six groups of 4 rats each were then kept on the standard vitamin E-deficient diet. Three groups were given 1 mg. of tocopherol per rat weekly, the other groups received no vitamin E. This treatment was continued for 98 days. During the last 18 days of this period doses of 1, 0·5 and 0·25 mg. of carotene were given to paired groups with and without tocopherol.

The carotene was purchased from The British Drug Houses, Ltd., and dissolved by warming in lard. A total volume of 1 ml. of carotene solution and lard was given mixed with a little solid diet before the main ration was fed; it was always completely eaten. Since the carotene was not highly purified and showed some fading during the period of the administration the amounts of pigment actually received by the rats must have been smaller than those calculated. Any effects due to this cause must, however, have been equalized between the paired groups at each level.

Table 5. *The effect of* dl-α-*tocopherol on the conversion of carotene into vitamin A*

Exp. 5

	−tocopherol					+tocopherol				
	Wt. of rat g.	Wt. of liver g.	Discoloration of uterus	I.U. of vitamin A Total	I.U. of vitamin A Per g. liver	Wt. of rat g.	Wt. of liver g.	Discoloration of uterus	I.U. of vitamin A Total	I.U. of vitamin A Per g. liver
1 mg. of carotene daily	163	7·5	+ + +	600	80	178	9·3	+	825	89
	154	10·0	+ + +	750	75	178	8·8	+ +	825	94
	151	8·0	+ + +	750	94	189	9·0	+	900	100
	168	10·2	+ + +	980	96	167	8·5	0	1050	123
Means	159	8·9		769	86	178	8·9		900	101
0·5mg. of carotene daily	161	9·4	+ + +	225	24	198	8·6	+ +	337	39
	138	8·0	+ + +	225	28	178	7·0	+	410	59
	162	7·5	+ + +	225	30	199	9·8	+	413	42
	168	9·7	+ + +	300	31	161	8·4	0	413	49
Means	157	8·7		244	28	184	8·5		394	47
0·25 mg. of carotene daily	131	7·6	+ + +	38	5	167	7·6	+ +	38	5
	146	7·5	+ + +	75	10	183	8·5	+ +	94	11
	158	7·5	+	113	15	175	7·8	+	132	17
	168	9·4	+ + +	131	14	206	9·9	+	169	17
Means	151	8·0		89	11	185	8·5		108	13

0 = normal. + = slightly discolored. + + = pale brown. + + + = medium, or deeper brown.

The vitamin A reserves determined at autopsy were found to be graded between the groups receiving different levels of carotene without a single instance of overlapping. This applied equally to groups with and without tocopherol. The differences in the vitamin A reserves ascribable to the administration of tocopherol were much less marked than in the previous experiments on the storage of preformed vitamin. Although slight, however, the differences were always in favour of the groups which had received tocopherol. The most marked difference between vitamin A reserves was found in the paired groups receiving 0·5 mg. of carotene daily. The mean total reserve for the group receiving tocopherol was 62% greater than the corresponding value for the untreated group.

In Table 5 it may be noted that in some of the rats which had been given tocopherol the uteri were slightly discolored. This may be attributed to the length of the period of the modified basal diet before the commencement of dosing with tocopherol. A rough superficial correlation between the height of the reserve and the freedom of the uterus from discoloration may be traced, and if data relating to rats with decidedly pale brown uteri are omitted in calculating the means the differences between the corresponding groups are slightly increased.

The storage of vitamin A in rats after the curing of prolonged avitaminosis-E (Exp. 6). Martin & Moore [1939] reported that rats which have been subjected to prolonged deficiency of vitamin E may sometimes be restored to good general condition, but not cured of paralysis, by treatment with large amounts of wheat germ. Two male rats were given the standard diet deficient in vitamin E for 12 months. The animals at this period were in a state of advanced avitaminosis-E, with paralysis. Their body weights were 184 and 180 g. After 60% of wheat germ had been added to the diet for a further 12 months the general condition of the rats had much improved, and the body weights were increased to 220 and 218 g., although the paralysis was little affected. The rats were then killed and found to have total vitamin A reserves of 30,000 and 38,000 I.U. respectively, or 2300 and 2900 I.U. per g. of liver. In the absence of evidence as to the extent of the reserves of companion animals killed before the commencement of the cure, no definite conclusion can be drawn from these results. It appears very probable, however, that treatment with wheat germ restored to normal the power of the liver to store vitamin A. It has been shown in a previous paper [Moore & Rajagopal, 1940] that even the inclusion of 60% of wheat germ in a diet otherwise free from vitamin A does not allow the accumulation of measurable stores of vitamin A in the liver.

Discussion

In all the above experiments in which halibut liver oil was given the vitamin A reserves were always much lower in rats deprived of vitamin E than in animals subjected to exactly the same treatment except for the addition of vitamin E. The degree of disparity varied in different experiments. In Exp. 1 the reserves of rats given vitamin E were about twice as high as those of deficient animals; in Exps. 2 and 3 about ten times, and in Exp. 4 about three times as high. No explanation of these differences in the degrees of disparity can at present be offered. In work by Bacharach [1940] confirming this effect of vitamin E deficiency in lowering the vitamin A reserves the differences noted between the treated and untreated groups were much smaller, amounting to only some 50% difference between means in favour of the treated group. In his work however the rats were kept on the deficient diet for a shorter period.

The efficiency of storage of the ingested vitamin A, neglecting the small contribution of carotene in the groups receiving wheat germ oil concentrate, may be calculated as follows:

% of ingested vitamin A found in the liver

	−E		+E
Exp. 1	20		46
Exp. 2 normal casein only	4·2		42
Exp. 3 normal casein only	5		50
Exp. 4 no tocopherol	14	3 mg. tocopherol	40

In the groups given vitamin E the rate of storage of 40–50% points to high efficiency, particularly in view of the prolonged periods during which the earlier doses of vitamin A must have been kept in the livers before they were examined. Without vitamin E the efficiency of storage in Exps. 2 and 3 was very poor. It must be pointed out that both in the present work and in that of Bacharach [1940] the dietary intake of vitamin A was large, and that even in the groups deficient in vitamin E the reserves of vitamin A were not very low in an absolute sense. Experiments at lower levels of dietary intake of vitamin A are obviously needed.

In the experiment on the conversion of carotene the differences between the groups treated and not treated with vitamin E were comparatively small, but greater differences might have been found if the duration of the experiment had been extended and if dosing of the control group had been started earlier.

The cause of the low vitamin A reserves found in vitamin E deficiency is obscure. It may be recalled, however, that the storage of vitamin A in the human liver may be greatly reduced in certain diseases, and notably in chronic nephritis [Moore, 1937]. The association of the reduced power to store vitamin A with the kidney injury which is always found after prolonged deficiency of vitamin E [Martin & Moore, 1936; 1938; 1939] may therefore be significant. The possibility that vitamin A might be lost in the urine of the deficient rats, as in the human subject in chronic nephritis, was investigated, but with negative results. It may be noted, however, that the rat does not seem to excrete vitamin A in its urine in any circumstances, whereas the dog, as I have been able to confirm, excretes the vitamin even when in normal health [Catel, 1938, 1, 2]. The apparent discrepancy may therefore be due to difference of species.

The question of what factors other than vitamin E deficiency may depress the vitamin A reserves is obviously of great interest. Since in the present work it was found that deficiency of protein caused at the most only a slight depression of the vitamin A reserves it is obvious that the phenomenon is not a general non-specific effect of malnutrition. Dann & Moore [1931] failed to detect any marked depression of the vitamin A reserves in rats kept for many weeks on diets deficient in the vitamin B complex. In hens deficient in vitamin K Tomaszewski & Engel [1939] found that neither the vitamin A nor vitamin C reserves were lowered. On the other hand, it is obvious that vitamin E deficiency is by no means unique in depressing the vitamin A reserves. Goerner [1937] has shown that the injection of carcinogenic agents into rats may cause marked reduction in the vitamin A reserves of the liver. The possibility of injury of the liver of the rat in vitamin E deficiency must be kept in mind in view of the observations of Barrie [1939], but no consistent evidence of liver injury was found in the present work.

The reduction of vitamin A reserves in vitamin E deficiency may afford an explanation of the rapid dispersal of the vitamin A reserves in rats observed in one particular experiment by Davies & Moore [1934]. Numerous attempts by the authors to repeat this experiment have given most inconsistent results, and it seems probable that the rapid loss of vitamin A in the experiment reported may have been due to an unsuspected deficiency of vitamin E in the basal diet. Preliminary experiments have indicated that the vitamin A reserves of rats deficient in vitamin E fall rapidly when the animal is restricted to a diet deficient in both vitamins A and E, but detailed publication of the results may await the completion of controlled experiments.

Summary

1. The vitamin A reserves of rats kept for prolonged periods on a diet deficient in vitamin E in which vitamin A was supplied as halibut liver oil were always much lower than those of control animals receiving supplements of vitamin E.

2. After dosing with carotene, the differences between the amounts of vitamin A formed by rats treated and not treated with vitamin E were relatively slight. This may have been due in part to the particular experimental conditions adopted, which resulted in the differentiation between adequacy and deficiency of

vitamin E being less clear cut than in the experiments on the storage of preformed vitamin A.

My thanks are due to Dr L. J. Harris for his valuable criticism and to Mr A. W. Davies for technical assistance.

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157. EFFECT OF A HIGH-FAT DIET ON THE EXCRETION OF BISULPHITE-BINDING SUBSTANCES IN THE URINE OF RATS DEFICIENT IN VITAMIN B_1

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(*Received 29 July 1940*)

THE first indication that rats can be maintained on a low intake of vitamin B_1 provided that their ration contains a high proportion of fat was given by Evans & Lepkovsky [1928]. Since then a number of experiments have been done on the effect of fat in delaying the onset of beri-beri or polyneuritis. It has been found that even synthetic fats have this characteristic action.

In earlier work in this laboratory [Banerji & Harris, 1939], it was observed that rats on a diet deficient in the vitamin excreted large amounts of bisulphite-binding substances in the urine, and that the extent of the abnormality was proportional to the degree of the severity of the deficiency. It was therefore thought worth while to investigate the effect of high-fat diets on this feature of the avitaminosis. Another abnormality which has been intensively studied in this Institute is the bradycardia seen in the deficient rat [e.g. Drury & Harris, 1930; Birch & Harris, 1934] and the opportunity was therefore also taken to study the effect of the high-fat diets on the heart rate.

EXPERIMENTAL

The bisulphite-binding substances in the urine were measured according to the method of Clift & Cook [1932]. In one of the experiments, however, 5 ml. of 15% Na_2HPO_4 solution were used, in place of 5 ml. of saturated $NaHCO_3$ solution, as the alkali for breaking down the bound bisulphite. This small modification in the method, which has been adopted for later work, was found to give a more sharply defined end-point without causing any alteration in the titration value. The rest of the procedure was exactly as described in the paper cited above.

Heart rates were taken with the electrocardiograph in the way described by Drury *et al.* [1930] and Birch & Harris [1934].

Table 1

	High-fat diet	Standard basal diet
Sucrose	—	60
Lard	75	—
Arachis oil	—	15
Casein (light white)	20	20
Salt mixture	5	5
Autoclaved marmite*	12	12
Cod-liver oil	2 drops daily	2 drops daily

Rats receiving the high-fat diet in Fig. 1 had 10% autoclaved marmite instead of 12%.

* 1½ atmospheres at *p*H 8–9.

In all the experiments the rats used were young males weighing from 60 to 90 g. Six rats were taken for each experiment, one group of three receiving a high-fat diet, and the remaining three receiving a standard basal diet [Birch & Harris, 1934] and serving as controls (Table 1).

The animals were housed in separate cages throughout to reduce complications from coprophagy or refection.

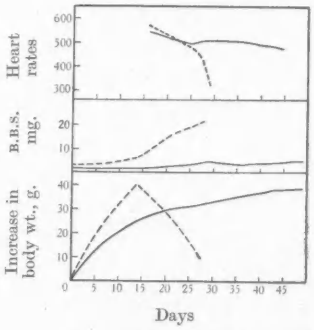

Fig. 1. Effect of high-fat diet on growth, excretion of bisulphite-binding substances and heart rate in rats deficient in vitamin B_1. Each curve shows the average for three animals. Composition of diets as in Table 1. —— Rats receiving high-fat diet. – – – Rats receiving standard basal diet.

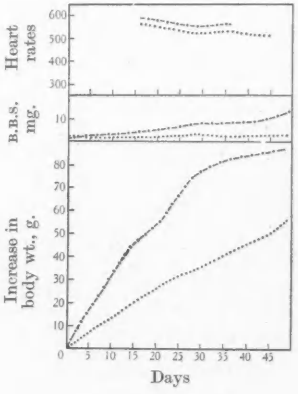

Fig. 2.

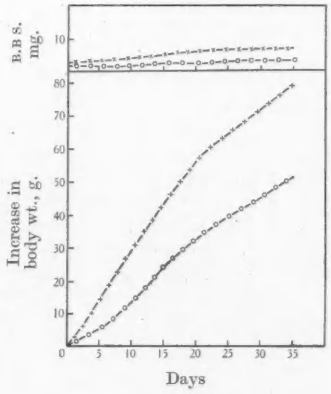

Fig. 3.

Fig. 2. Effect of high-fat diet, during partial deficiency of vitamin B_1 (5 μg. per rat per day), on growth, excretion of bisulphite-binding substances, and heart rate. Each curve is the average for three rats. ····· Rats receiving high-fat diet + 5 μg. aneurin daily. ·—·— Rats receiving standard basal diet + 5 μg. aneurin daily.

Fig. 3. Effect of high intake of fat with nearly optimum allowances of vitamin B_1 (10 μg. of aneurin daily) as compared with normal intake of fat. —o— Rats receiving high-fat diet + 10 μg. aneurin daily. —×— Rats receiving standard basal diet + 10 μg. aneurin daily.

Results

1. *Effect of high-fat diet in the absence of vitamin B_1*

It will be seen from Fig. 1 that the gradual increase in the excretion of bisulphite-binding substances in the urine, from 2 mg. to 20 mg., which occurred in the animals on the standard basal diet without vitamin B_1, did not occur in the animals taking a high proportion of fat. The control rats showed an initial gain in weight and then a fall, ending with the death of the animals after about 30–35 days, whilst those on a high-fat diet gained weight slowly but continuously throughout the experimental period. Lastly, whereas the control animals developed the bradycardia typical of aneurin deficiency, those on the high-fat intake showed no significant changes in their heart rate. Three similar experiments gave substantially the same results.

2. *Effect of addition of supplements of aneurin*

The effect of the provision of graded supplements of aneurin was then studied. Each rat received daily additions of aneurin, amounting to 5μg. (Fig. 2) or 10μg. (Fig. 3). The aneurin was injected intraperitoneally.

The addition of 5μg. aneurin resulted in a very slow rise in the bisulphite-binding substances in the control rats, the values reached being far less than those attained in the absence of aneurin. In the high-fat group the excretion showed no rise. Both groups showed a continuous gain in weight, but this was less in the animals on the high-fat diet. The heart rates remained normal in both groups. This experiment was repeated a second time with the same result.

Daily supplements of 10μg. aneurin almost completely suppressed the rise of bisulphite-binding substances in the control groups, which, as with the lower supplement, showed a more rapid gain in weight than the group taking the high-fat diet.

Discussion

The above results show that the consumption of a diet rich in fat is able to counteract, in a quantitative manner, two of the abnormalities associated with vitamin-B_1 deficiency, or partial deficiency, in the rat—namely the increased excretion of bisulphite-binding substances and the slow heart rate. The high intake of fat not only alleviated these two symptoms of the deficiency, but apart from a slightly depressing effect on the growth did not appear to have any injurious action upon the rat. This latter point seems of interest because there can be no doubt that in the human subject so high an intake of fat would have given rise to a ketosis. In the rat, on the contrary, so far from producing ketosis, the fat counteracted the excretion of acetone-bodies resulting from the vitamin deficiency. The reason for the apparent difference between humans and rats is not clear and is being made the subject of further experiments.

Some authors have claimed that the delay in the onset of symptoms of deficiency brought about by the consumption of large amounts of fat is probably due to the aneurin-carrying capacity of the fat. As against this, it has been proved that the esters of some of the fatty acids exert a sparing action while others do not. Salmon & Goodman [1937] have claimed that the effectiveness of the esters of fatty acids regarding their sparing action is maximum with 8-carbon acid and decreases in both directions.

Whether lard contains any aneurin has been investigated recently by Melnick & Field, Jr. [1939], using their own chemical method of estimation.

They concluded that lard is devoid of aneurin. The lard used in the above experiments was examined several times for its aneurin content in this laboratory by the thiochrome method. No aneurin was detected on any occasion.

Whether there is any stored aneurin or cocarboxylase in the tissues of the rats receiving aneurin-free high-fat diets requires further study, and experiments are now in progress.

The results of these experiments have an important bearing on the whole question of the methodology used for the biological assay of vitamin B_1. As we have seen, fat spares the more specific symptoms of deficiency such as the excretion of bisulphite-binding substances, polyneuritis and bradycardia, but on growth the effect is initially one of retardation and only later does it prevent the loss of weight. It is therefore conceivable that the determination of the "antineuritic" value of a specimen rich in fat might give different results depending on whether the method used was a growth test or a more specific test such as the bradycardia method.

More important however is the bearing of these results on the biological as compared with the chemical estimation of vitamin B_1. The sparing action of fats on the symptoms of deficiency has, as already stated, led to the statement by many observers that lard for example contains the vitamin. Chemical tests on the other hand have failed to confirm this. It might be argued that, since one's concern with vitamins is in the end always biological, it is the "antineuritic" action of a substance which one desires to estimate rather than its actual chemical content of vitamin B_1.

It is clear, nevertheless, that the antineuritic action of a fatty substance in preventing the onset of polyneuritis or bradycardia may be influenced by the proportion of fat in it. In view of this and of possible species differences in the vitamin-sparing action of fats, the ideal method of expressing the vitamin B_1 activity of a diet would be to state the actual quantity of the vitamin as determined chemically, together with the composition of the diet in respect to fat, protein and carbohydrate, with possibly an indication also of the relative availability of the vitamin in the food in question. (There are indications for example that in fibrous and bulky foods which are not always readily digested, the vitamin is sometimes less readily absorbed.) The method of expressing antineuritic potency as not necessarily identical with vitamin B_1 content would resemble the customary procedure in indicating the antirachitic activity of a diet by stating the number of units of vitamin D together with such factors as the $Ca\text{-}PO_4$ ratio and the acid-base balance of the diet.

Finally, these results are of interest in connexion with the proposed use of a carbohydrate tolerance test (cocarboxylase function) for detecting hypovitaminosis-B_1, in the human subject. The experiments with rats emphasize once again the need, already indicated in an earlier paper [Banerji & Harris, 1939], for adequately controlling the basal diets of individuals submitted to such tests. Actual experience of tests on human beings has indicated for example that there may occur a rapid increase in B.B.S. in the urine almost at the very beginning of a period of restriction to a diet of polished rice, and this cannot be attributed solely to the low intake of vitamin B_1. As mentioned by Banerji & Harris [1939] the most suitable conditions for applying a cocarboxylase function test are not the same for the human as for the rat. With rats the administration of lactate and estimation of excretion of pyruvate or B.B.S. is found to be a sufficiently satisfactory procedure. With human beings on the other hand the direct administration of pyruvate seems preferable. This work on human beings is still

in progress and will be discussed in a later paper, but it seems advisable to allude here to the differences in technique, as between the human and the rat, to avoid the possibility of misunderstanding.

Summary

The ingestion of a high-fat diet by rats deprived of vitamin B_1 diminished the excretion of bisulphite-binding substances in their urine and alleviated the severity of the bradycardia. The aneurin-sparing action of fat, therefore, is not limited to its effect in protecting such animals against loss of weight or the development of the better known symptoms of deficiency, but shows itself likewise in the absence of these two characteristic signs of the avitaminosis. The significance of these results for vitamin assays, and for the assessment of deficiency, is discussed.

The author wishes to take this opportunity to express his gratitude to Dr L. J. Harris for his continual interest and valuable criticisms.

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That this is the explanation of the apparent inhibition obtained by previous workers is clearly demonstrated by further experiments in which the effects of cyanide and thiosulphate are compared.

In another series of experiments the estimation of peroxide and of oxidation of iodide were carried out in media: (1) untreated, (2) dialysed, (3) boiled and (4) mixture of boiled and dialysed after standing for 24 hr. The iodide oxidations were only allowed to proceed for 24 hr. The results of these experiments (Table 3)

Table 3. *Estimation of H_2O_2 and iodide oxidation*

H_2O_2 in μl. of O_2 and iodide oxidation in μl. of O_2 equivalent in 10 ml. of culture medium (untreated, dialysed, boiled and mixture of boiled and dialysed media). The peroxide in the mixture of boiled and dialysed media was determined after standing for 24 hr.

	Untreated		Dialysed		Boiled		Boiled and dialysed	
Flask	H_2O_2	Iodide	H_2O_2	Iodide	H_2O_2	Iodide	H_2O_2	Iodide
1	60·8	16·8	0	1·34	60·8	0	60·8	8·06
2	41·2	10·75	0	0	41·2	0	56·6	6·05
3	52·0	12·1	0	0	52·0	0	62·4	5·38
4	20·33	16·8	0	0	30·5	5·37	60·9	16·8
5	36·13	16·8	0	0	51·6	0	56·7	27·55
6	52·13	30·2	0	0	62·6	2·69	62·6	34·6

clearly show that at least two factors are involved in iodide oxidation: one is heat-labile and the other is dialysable. Further, the high peroxide concentrations in the mixtures of boiled and dialysed media indicate that there must be a source of peroxide in the medium. Thus, the fact that the iodide oxidation could exceed in its equivalent the amount of peroxide present in the medium before addition of iodide, could no longer exclude the participation of peroxide. This was ultimately confirmed by the experiments carried out in the presence and absence of catalase. For this purpose, four flasks of medium were prepared as before except that after sterilization and before inoculation 1 ml. of sterilized catalase was added to two of the flasks. The catalase was sterilized by precipitation with absolute alcohol, and redissolved in sterile distilled water. The following estimations were carried out on these media: (1) peroxide in 10 ml. of media, (2) iodide formed in 24 hr. in 10 ml. of media in the presence and absence of catalase and (3) in the presence of $10^{-3}M$ cyanide added to media devoid of catalase.

The results of these experiments (Table 4) show that catalase added either before or after incubation entirely destroys the iodide-oxidizing activity of the

Table 4

KI oxidation per 24 hr., and H_2O_2 concentration expressed as μl. O_2

	No catalase		Catalase	
Flask 10 ml. of media	1	2	3	4
H_2O_2	30·25	110·3	0·0	0·0
KI oxidation	11·4	13·4	0·0	0·0
KI oxidation in $10^{-3}M$ KCN	33·6	40·3	—	—
KI oxidation after catalase addition	0·0	0·0	—	—

medium. This clearly demonstrated that the oxidation is due entirely to the reaction: $H_2O_2 + 2HI = 2H_2O + I_2$.

The O_2 uptake by culture medium

Examination of samples of medium in Barcroft manometers reveals the presence of an oxidizing system with an O_2 uptake of about 45 μl. per hr. per ml. If all the O_2 absorbed were reduced to peroxide, the latter would be sufficient to account for the iodide oxidations already observed. The effect of KI on the O_2 uptake was examined and is shown in Fig. 1. The free iodine formed was titrated with thiosulphate and was found to vary directly as the square of the initial iodide concentration (Fig. 2) which forms additional evidence for the reaction $H_2O_2+2HI=2H_2O+I_2$, since H_2O_2 may well be kept at a fairly constant value by the enzyme system.

That the inhibition of O_2 uptake by KI in these experiments is due not to the iodide itself but to the free iodine formed was easily shown by the fact that the addition of $Na_2S_2O_3$ removes this inhibition (Fig. 3).

A more striking result was obtained in the presence of $M/170$ KCN instead of thiosulphate. The cyanide not only prevented the accumulation of free iodine, but by inhibiting all traces of catalase made the total peroxide formed in the medium available for the oxidation of iodide, with the result that the O_2 uptake was almost double that of the medium alone (Fig. 4).

The primary oxidation system in the medium and origin of H_2O_2

It can be definitely stated now that the oxidation of iodide in the culture medium is a secondary oxidation reaction due to H_2O_2 formed as a result of the activity of the primary oxidation system present in the medium. This is strongly supported by the results of experiments related in previous sections which can be summarized as follows:

(1) The oxidation of iodide in the culture medium depends on two kinds of factors, (*a*) thermolabile and non-dialysable—the enzyme, and (*b*) thermostable and dialysable—the substrate and H_2O_2 formed in the primary reaction.

(2) The medium alone has a distinct O_2 uptake, which shows that it contains a complete oxidizing system, namely the enzyme and its substrate.

(3) The H_2O_2 is gradually formed in the medium exposed to air, which shows that the O_2 in the primary oxidation reaction is reduced to peroxide.

(4) The iodide oxidation in the medium is inhibited by the addition of catalase which destroys the H_2O_2.

(5) The O_2 uptake by the medium is doubled on the addition of iodide and cyanide, the latter acting only as inhibitor of catalase present in the medium.

The nature of the primary oxidation reaction and the mechanism of iodide oxidation

The simplest way of determining the nature of the primary oxidizing system consists in separating the enzyme from its substrate and in identifying the latter. For this purpose the culture medium obtained in the usual way was dialysed for at least 16 hr. against running tap water and after dialysis precipitated with five volumes of acetone. The precipitate was centrifuged off and redissolved in 10 ml. $M/5$ acetate buffer pH 5·6. The activity of this preparation tested with boiled medium used as a substrate shows a yield of at least 80 %.

In order to identify the specific substrate which is oxidized by this enzyme preparation, its catalytic activity was tested with about 20 different substances such as succinic, lactic, malic and glycollic acids, alanine, glutamic acid, uric acid, alcohol, acetaldehyde, glucose, fructose, p-phenylenediamine and different

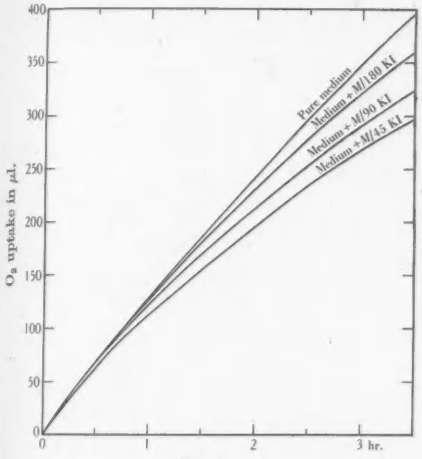

Fig. 1.

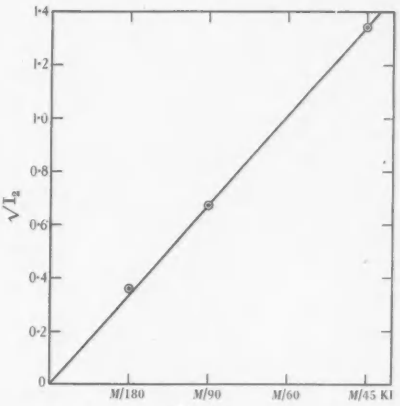

Fig. 2.

Fig. 1. The effect of iodide on the O_2 uptake of the medium.

Fig. 2. The relation between the concentration of iodide and the free iodine formed.

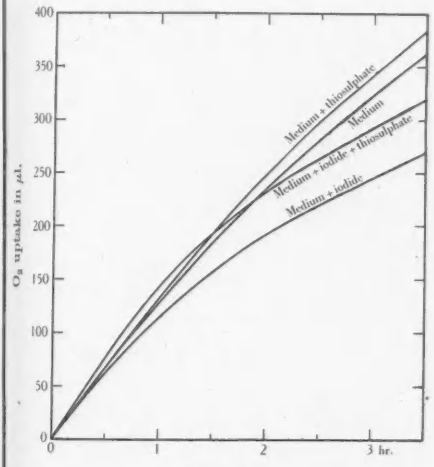

Fig. 3.

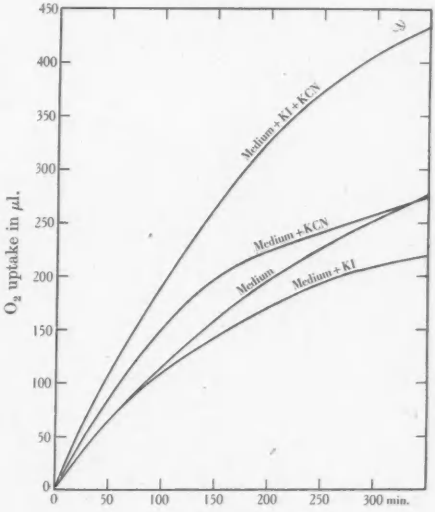

Fig. 4.

Fig. 3. The effect of iodide on the O_2 uptake of the medium in the presence of thiosulphate. 2·7 ml. medium; 0·4 ml. *M*/4 KI; 0·4 ml. *M*/40 $Na_2S_2O_3$.

Fig. 4. The effect of iodide on the O_2 uptake of the medium in the presence of cyanide.

phenols such as phenol, catechol, quinol, resorcinol, phloroglucinol. Of all these substances only glucose acted as a substrate for this enzyme preparation.

The enzyme responsible for the primary oxidation reaction in the medium can therefore be identified with the glucose oxidase of *A. niger* discovered and described by Müller [1928; 1929, 1, 2; 1931], reinvestigated by Franke & Lorenz [1937] and purified by Franke & Deffner [1939]. This view is strongly supported by further experimental results which can be summarized as follows:

(1) In absence of glucose the acetone preparation of the enzyme prepared from culture media has no O_2 uptake and does not oxidize iodide.

(2) In presence of $M/6$ glucose the preparation shows a marked O_2 uptake and iodide undergoes a secondary oxidation doubling the O_2 uptake of the enzyme-glucose system alone.

(3) This secondary oxidation of iodide is abolished on the addition of catalase.

(4) If in sucrose media the oxidation of iodide was due to the peroxide formed by the glucose oxidase system, it might be expected that in media having no source of glucose iodide would not undergo oxidation. Accordingly, the mould was cultivated on two fresh media, one containing galactose, the other fructose. After 68 and 44 hr. growth respectively, the media were filtered and to 5 ml. of each of them were added 50 mg. of KI. On allowing to stand for 48 hr. no oxidation of iodide was apparent. Further, when the media were examined manometrically the galactose medium showed no O_2 uptake and the fructose medium only a slight uptake of 0·7 μl. O_2 per ml. per hr.

(5) I_2 was found also to act as a hydrogen acceptor which was demonstrated by anaerobic experiments carried out in the usual way in Thunberg tubes. These experiments, summarized in Table 5, suggest that the decrease in the O_2 uptake

Table 5. *Reduction of I_2 by glucose oxidase ± glucose in absence of oxygen*

Thunberg tubes	Enzyme ml.	Acetate *p*H 5·6 ml.	$M/100$ I_2 ml.	$M/10$ glucose ml.	Water ml.	Time of decoloration min.
1	1·0	0·5	0·1	1·0	—	9–9·5
2	—	0·5	0·1	1·0	1·0	55–60
3	1·0	0·5	0·1	—	1·0	⩾60

by the system in presence of iodine is partly due to its competition with O_2 in acting as hydrogen acceptor. The reduction of iodine by this system explains also Korczewski's observation that during the oxidation of iodide to free iodine an equilibrium is reached which prevents the further accumulation of iodine in the medium.

(6) The enzyme system of the medium shares with the glucose oxidase and other oxidizing systems which reduce molecular oxygen to H_2O_2 the property of promoting secondary oxidation reactions. The addition of alcohol and catalase to such systems doubles their O_2 uptake. In this reaction, as was shown by Keilin & Hartree [1936], the oxidation of alcohol to aldehyde by H_2O_2 formed in the primary oxidation reaction is catalysed by small concentration of catalase. An increase in O_2 uptake by the media was also observed on addition of acetaldehyde which is oxidized to acetic acid. This secondary oxidation reaction, like that of iodide, is on the contrary inhibited by catalase. Using phosphate instead of acetate buffer, the formation of acetic acid can be easily demonstrated by the

lanthanum test. The secondary oxidation of the aldehyde was also followed manometrically and as shown in Fig. 5 the addition of acetaldehyde to the primary oxidation system doubles its O_2 uptake.

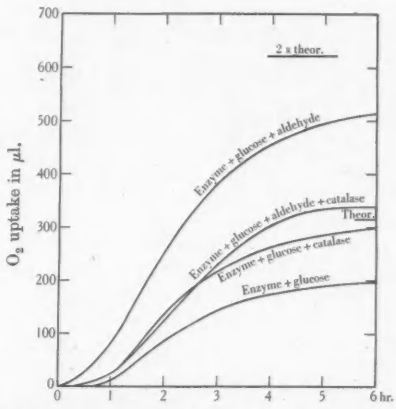

Fig. 5. The secondary oxidation of acetaldehyde by the glucose oxidase system in phosphate buffer. 5 mg. glucose; 30 mg. freshly prepared acetaldehyde.

(7) Finally the enzyme system of the medium, like Müller's glucose oxidase, is not inhibited by KCN, NaN_3, H_2S, NH_2OH or $Na_4P_2O_7$. On the contrary these reagents, as shown in Table 6, if anything accelerate the O_2 uptake of the system. On the other hand NaF was found to inhibit slightly and $NaHSO_3$ considerably the activity of this enzyme.

Table 6. *Effect of inhibitors on the O_2 uptake by culture media*

Expressed as % acceleration (+) or inhibition (−)

	%		%		%
M/250 NaN_3	+55	*M*/50 Na_2S	+86	*M*/50 NaF	−18·7
M/500 NaN_3	+47	*M*/50 $Na_4P_2O_7$	+11·1	*M*/100 NH_2OH	+23
M/1000 NaN_3	+29	*M*/50 $CH_2I.COONa$	+11·7	*M*/100 KCN	+28
		M/200 $NaHSO_3$ (*p*H 4·4)	−60		

All these experiments clearly demonstrate that there is no indication of the existence in the culture medium of *A. niger* of a special enzyme oxidizing iodide, as was postulated by Raciborski [1905] and Korczewski [1922]. It shows on the contrary that the oxidation of iodide in this medium is due solely to H_2O_2 formed in the primary oxidation of glucose catalysed by the glucose oxidase of Müller which diffuses out from the mycelium of the mould into the culture medium. Moreover, the secondary oxidation of iodide can be obtained in the presence of every primary oxidation system which like glucose oxidase or xanthine oxidase systems reduces molecular O_2 to H_2O_2, provided that the peroxide does not undergo destruction by catalase or by acting as hydrogen acceptor.

SUMMARY

1. The culture medium of *Aspergillus niger*, as was shown by previous workers, oxidizes iodide to free iodine.

2. This oxidation is not however catalysed by a specific iodide oxidase or by an oxygenase as was suggested by previous workers, but by hydrogen peroxide formed in the medium.

3. This peroxide is formed as a result of a primary oxidation reaction of glucose catalysed by glucose oxidase.

4. The oxidation of iodide is therefore a secondary oxidation reaction which can be promoted by every primary oxidizing system which reduces molecular oxygen to H_2O_2.

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158. THE VITAMIN B_2 COMPLEX OF LIVER: THE IDENTITY OF THE LIVER FILTRATE FACTOR WITH PANTOTHENIC ACID

By B. LYTHGOE, T. F. MACRAE, R. H. STANLEY, A. R. TODD and C. ELIZABETH WORK

From the Chemistry Department, University of Manchester and the Division of Nutrition, Lister Institute

(*Received 19 August 1940*)

In a previous communication [Macrae *et al.* 1939] we described the preparation from liver extracts of highly potent concentrates of a nutritional factor for rats to which we applied the name "liver filtrate factor" [cf. Edgar *et al.* 1938]. At that time we drew attention to the similarity in properties between this factor and pantothenic acid [Williams *et al.* 1938; 1939] and discussed the possibility that the two might ultimately prove to be identical. Pantothenic acid was then known to be the β-alanide of a readily lactonizable hydroxy-acid of unknown constitution and had been shown to be identical with the chick antidermatitis factor [Woolley *et al.* 1939, 1, 2; Jukes, 1939]. The suggestion that this acid was concerned in rat growth was supported to some extent by other evidence; for example, impure concentrates of pantothenic acid were shown to possess growth-stimulating properties for rats [Subbarow & Hitchings, 1939; Oleson *et al.* 1939].

Preliminary experiments showed that the active material present in our concentrates contained an amide linkage. Using micro-titration methods of the Willstätter-Waldschmidt-Leitz type it was found that destruction of the factor by hydrolysis with dilute alkali caused an increase in the titre of the acid material with alcoholic NaOH, whilst the titre after hydrolysis with dilute acid remained unchanged; this is in agreement with the production during hydrolysis of a readily lactonizable acidic fragment. It was further found that β-alanine could be isolated from hydrolysed concentrates of our factor as its β-naphthalene-sulphonyl derivative, although from the mode of preparation no free β-alanine could have been present in the unhydrolysed concentrate. When the lactone portion from these hydrolysed concentrates (itself completely inactive in rats in daily doses of about 2 mg.) was coupled with β-alanine, using the method employed by Woolley *et al.* [1939, 2] for regeneration of the chick anti-dermatitis factor, the reconstituted material was found to be fully active when fed to rats in daily doses of about 1 mg. These results correspond with those obtained by Woolley *et al.* [1939, 1, 2] with the chick factor. We also commenced, in common with many other investigators [Subbarow & Rane, 1939; Babcock & Jukes, 1940; Reichstein & Grüssner, 1940*], the synthesis of β-alanides of various dihydroxyvaleric acids, partly in the hope of obtaining an active substance but more especially with a view to studying the chemical behaviour of mixtures of these compounds. Of the compounds prepared *γ:δ-dihydroxyvaleryl-β-alanine* has not hitherto been described; details of the preparation of its methyl ester are given in the experimental section.

* Through the kindness of Prof. Reichstein we were privileged to see the manuscript of a paper on this subject which was submitted to the *Helvetica Chimica Acta* May 1940. The actual published paper we have not up to the present been able to obtain.

While these experiments were in progress the identification of pantothenic acid as α:γ-dihydroxy-β:β-dimethylbutyryl-β-alanine and its synthetic preparation were announced in an important communication by Williams & Major [1940]. Through the kindness of Dr Major of Merck and Co., Inc., to whom we are deeply indebted, we were enabled to test the activity of a specimen of the synthetic material on our rats. The optimal daily dose for a rat we found to be contained in about 50 μg. of the synthetic dextrorotatory material, this supplement supporting, under our experimental conditions, a growth of about 16 g. per week, equivalent to the response evoked by our liver filtrate factor. Rats receiving a daily supplement of 100 μg. pantothenic acid plus additional liver filtrate factor did not increase in body-weight significantly more rapidly than did animals receiving pantothenic acid or liver filtrate factor alone, i.e. liver filtrate factor and pantothenic acid can replace each other but have no supplementary effect and are therefore biologically identical. It would therefore appear that our purest preparation of liver filtrate factor, which was active in a daily dose of 180 μg. [Macrae *et al.* 1939], must have contained about 25% pantothenic acid.

Evidence has already been published from these laboratories [El Sadr *et al.* 1939] that our crude liver extracts contain at least three factors of the vitamin B_2 complex which are required to ensure maximal growth in rats receiving as supplements the already well-established members of this complex, viz. riboflavin, nicotinamide and vitamin B_6. The first of these unknown factors, which we have been accustomed to designate for our own convenience "factor α" or "liver filtrate factor", is characterized by its extractability from acid aqueous solutions by amyl alcohol, its failure to be adsorbed by fuller's earth at acid reactions, and its lability to acid and alkali; the identity of this factor with pantothenic acid has been fully established in this paper. Our second factor—"factor β"—is contained in that portion of liver extract which, at *p*H 1, is neither adsorbed by fuller's earth nor extracted with amyl alcohol. That it is a single growth factor we have as yet no definite proof, but its biological entity cannot be doubted. When it is supplied to rats receiving optimal supplements of factor α (pantothenic acid) a markedly higher growth-rate is observed. Since these results were published papers from other laboratories have appeared which afford further independent evidence of the existence in liver and other materials of a factor distinct from pantothenic acid which is not extracted from acid aqueous solutions by organic solvents [e.g. Hitchings & Subbarow, 1939; Mohammad *et al.* 1940; Hoffer & Reichstein, 1939; Kringstad & Lunde, 1939; Black *et al.* 1940]. It is this material to which Black *et al.* [1940] now apply the term "factor *W*", a designation formerly applied to a mixture of factors which included pantothenic acid and vitamin B_6.

We have now obtained further evidence that our third factor—factor γ—is a biological entity. Rats receiving, as source of the unidentified B_2-vitamins, acid-autoclaved whole extract of liver together with concentrates of factor α and factor β show a growth rate of about 35–40 g. per week as compared with about 20 g. per week with acid-autoclaved whole extract of liver, 15 g. per week with concentrates of factor β and about 30 g. with concentrates of both factors α and β. There must therefore be at least one further factor (γ) present in acid-autoclaved whole liver extracts. Details of these experiments will be published later. Whether these three factors α, β and γ are together sufficient to produce optimal growth in rats receiving supplements of aneurin, riboflavin and vitamin B_6 we wish at present to leave an open question. Experiments designed to test this and to effect the purification and isolation of factors β and γ are at present in progress.

Experimental

Titration experiments

The results of a series of microtitration experiments on a typical liver filtrate factor concentrate (concentrate 45, containing 1 rat day dose in 0·72 mg.) are set out in Table 1.

Table 1

	Wt. of concentrate mg.	Total NaOH required for neutralization (g.-equivalents $\times 10^{-5}$)
Untreated concentrate, titrated in aqueous solution	6·773	3·16
Untreated concentrate, titrated in alcoholic solution	6·773	3·29
Concentrate hydrolysed by alkali, the hydrolysis products titrated in aqueous solution	6·773	3·40
Concentrate hydrolysed by alkali, the hydrolysis products titrated in alcoholic solution	6·773	5·43
Concentrate hydrolysed by acid, the hydrolysis products titrated in aqueous solution	6·773	2·64
Concentrate hydrolysed by acid, the hydrolysis products titrated in alcoholic solution	6·773	3·33

These results lead to the following conclusions: (*a*) the concentrate contains a mixture of acidic materials with an approximate mean equivalent of 214; (*b*) the very small difference between the titre of the concentrate in aqueous and alcoholic solutions indicates the presence of only small quantities of basic nitrogen; (*c*) the titre of the concentrate after hydrolysis with alkali shows a large increase over that of the original concentrate when titration is carried out in solution in alcohol of concentration $>97\%$, but little increase is noted if the titrations are carried out in aqueous solution; this indicates the presence in the concentrate of material of an amide nature, the basic portion liberated from it on hydrolysis being of the amino-acid type; (*d*) the acidic portion of the amide material must be readily lactonizable, since on hydrolysis with acid the titre of the product in aqueous solution shows a decrease, whereas that in alcoholic solution remains unchanged.

Isolation of β-naphthalenesulphonyl-β-alanine

A portion (2·4 g.) of liver filtrate factor concentrate 45 (containing 1 rat day dose in 0·72 mg.) was heated to 100° for 2 hr. with *N* H_2SO_4 (100 ml.). After cooling, the solution was extracted 6 times with ethyl acetate (100 ml.). The aqueous acidic phase was freed from sulphate ions with barium hydroxide, evaporated to dryness and treated with conc. HCl (30 ml.). Excess acid was removed *in vacuo* and the dry residue extracted with 95% ethyl alcohol (2×100 ml.); the alcoholic extracts on evaporation yielded an oil. This was taken up in water (200 ml.) and freed from chloride ions by shaking for 1 hr. with a suspension of silver oxide (from 15 g. $AgNO_3$), the excess of silver ions then being removed by H_2S. After filtration the solution was concentrated to 8 ml. and a portion (2 ml.) treated with alkali and shaken with an ethereal solution of β-naphthalenesulphonyl chloride (250 mg. in 15 ml. ether) as described by Weinstock *et al.* [1939]. On acidification of the aqueous layer β-naphthalenesulphonyl-β-alanine separated as white plates which after 3

recrystallizations from hot water had M.P. 134·5–135·5°. An authentic specimen in the same bath had M.P. 135·5–136·5° and a mixture of approximately equal amounts of both materials melted at 135·5–136·5°. (Found: C, 55·7; H, 4·7; N, 4·9%; calc. for $C_{13}H_{13}O_4N$: C, 55·8; H, 4·7; N, 5·0%.).

Reconstitution of pantothenic acid

The method used was analogous to that described by Woolley *et al.* [1939, 2]. Liver filtrate factor concentrate 48 (13·08 g.) was heated to 100° with *N* H_2SO_4 (200 ml.) for 2 hr. After cooling, the solution was adjusted to *p*H 7·5 with $NaHCO_3$ and lactonic material was extracted with ethyl acetate (6 × 100 ml.). The dry weight of the extracted material was 2·80 g. A portion (1·231 g.) was heated with *N* NaOH (15 ml.) at 100° for 1 hr., the solution evaporated to dryness and the residual solid heated to 97° with acetic anhydride (30 ml.) for 1 hr. Excess acetic anhydride was then removed under reduced pressure and the acetylated material was treated with thionyl chloride (45 ml.) at room temperature for 1 hr.; excess of the reagent was then removed under reduced pressure. The residue was taken up in dry pyridine (10 ml.), freshly prepared β-alanine ethyl ester (6 g.) added and the mixture left at room temperature for 1 hr., after which the pyridine was removed under reduced pressure. The residual material was taken up in water (100 ml.), acidified to *p*H 1 with H_2SO_4 and extracted with ethyl acetate (6 × 100 ml.). On evaporation the extracts yielded a brown oil which was hydrolysed by standing for 1 hr. at room temperature in alcoholic NaOH (2 g. NaOH in 70 ml. ethyl alcohol). Excess alkali was neutralized with alcoholic HCl and, after removal of NaCl by filtration, the solution was evaporated, leaving a brown oil. The results of the biological test on this material together with the tests on the lactone fraction and the original liver filtrate factor concentrate are recorded in Table 2 below.

Biological tests

The method used for the estimation of liver filtrate factor differed from that previously described [Edgar *et al.* 1938] only in that crystalline synthetic vitamin B_6 was used as a supplement instead of a liver or yeast fuller's earth eluate containing that vitamin. The young rats received our cooked starch diet and as

Table 2. *Growth response of rats receiving, as sources of B-vitamins, aneurin, riboflavin and vitamin B_6, to calcium* d-*pantothenate, to the lactone fraction of hydrolysed liver filtrate factor and to that fraction coupled with β-alanine*

Daily supplement given	No. of rats	Average weekly wt. increase in g. of groups during 2 weeks subsequent to giving supplement
None	5	6·2, 5
1 rat day dose liver filtrate factor	4	17·8, 15
100 μg. Ca *d*-pantothenate	3	14, 17
50 μg. Ca *d*-pantothenate	1	18, 16
25 μg. Ca *d*-pantothenate	1	6, 6
100 μg. Ca *d*-pantothenate + 1 rat day dose liver filtrate factor	2	20, 17
0·98 mg. lactone fraction*	2	8, 8·5
1·96 mg. lactone fraction	1	7, 3
0·98 mg. lactone fraction coupled with β-alanine	2	16, 14

* Corresponds to 1 rat day dose of unhydrolysed concentrate.

sources of B-vitamins each animal was given daily 10–15 μg. aneurin, 50 μg. riboflavin and 10 μg. vitamin B_6. After 2–3 weeks on this diet the growth rate of the animals had decreased to about 6 g. per week, and at this stage the test doses were given; when an active dose of liver filtrate factor was supplied the growth rate of the animals increased to 15–20 g. per week. We have continued to find the growth response on addition of liver filtrate factor so constant that reliable results were obtained when only two animals were employed for a single test material; where practicable, however, we have in most cases given the test materials to more than two animals.

The results of tests on relevant materials are given in Table 2. The amount of calcium *d*-pantothenate at our disposal was limited and permitted of only one animal being tested on the 25 and 50 μg. daily dose levels. The growth rates observed in these animals indicate, however, that the rat day dose of this factor is >25 μg. and is probably about 50 μg.

γ:δ-Dihydroxyvaleryl-β-alanine methyl ester

This was prepared by condensing allylacetyl chloride and β-alanine ethyl ester to give allylacetyl-β-alanine ethyl ester which was hydrolysed to *allylacetyl-β-alanine*. The latter was then oxidized in neutral solution with barium permanganate to *γ:δ-dihydroxyvaleryl-β-alanine* which was isolated as the methyl ester. Freshly prepared β-alanine ethyl ester (11 g.) in dry pyridine (30 ml.) was treated at 0° with allylacetyl chloride (11 g.) and the reaction mixture heated to 90° for 16 hr. The solid mass formed on cooling was extracted with ether and, after removal of the ether, the residue was acidified with *N* HCl (20 ml.) and the crude allylacetyl-β-alanine ethyl ester isolated with $CHCl_3$. Hydrolysis of this to the free acid was accomplished by letting it stand at room temperature with occasional shaking for 1 hr. with *N* NaOH (86 ml.) followed by neutralization with *N* HCl (86 ml.). The solution was evaporated under reduced pressure, the residue extracted with absolute alcohol and the extract evaporated. The product was dissolved in acetone and filtered from traces of β-alanine; evaporation yielded *allylacetyl-β-alanine* (6·9 g.) which after recrystallization from benzene-light petroleum had M.P. 70°. (Found: C, 55·8; H, 7·5; N, 8·4%; $C_8H_{13}O_3N$ requires C, 56·1; H, 7·7; N, 8·2%.) A portion of this material (6·23 g.) was neutralized with $Ba(OH)_2$ solution and dissolved in water (600 ml.); a solution of barium permanganate (6·79 g. of 98% $Ba(MnO_4)_2$ in 600 ml. H_2O) was added with stirring during 1 hr. The precipitated manganese dioxide was filtered off and Ba ions were removed by H_2SO_4. Evaporation *in vacuo* yielded a yellow oil which was extracted with absolute alcohol. The syrupy *γ:δ-dihydroxyvaleryl-β-alanine* obtained on evaporation of the alcohol failed to crystallize and attempts to obtain a solid benzyl-thiuronium derivative were unsuccessful.

The crude material was therefore dissolved in alcohol, treated with diazomethane and the resulting methyl ester purified by molecular distillation. *γ:δ-Dihydroxyvaleryl-β-alanine methyl ester* distilled at 80–90°/10^{-5} mm. as a colourless syrup which subsequently set to a hygroscopic crystalline mass M.P. 48–49°. (Found: C, 50·2; H, 7·9; N, 6·2%; $C_9H_{17}O_5N$ requires C, 49·3; H, 7·8; N, 6·4%.)

SUMMARY

1. Liver filtrate factor has been shown to be identical with pantothenic acid.

2. Evidence is presented that the factor in liver extracts which is neither adsorbed by fuller's earth nor extracted from acid aqueous solution by amyl

alcohol is a biological entity (factor β) and that a third unidentified factor (γ) is present in acid-autoclaved whole extract of liver.

3. A synthesis of γ:δ-*dihydroxyvaleryl*-β-*alanine methyl ester* is described.

The authors wish to thank the Department of Scientific and Industrial Research for a Maintenance Allowance held by one of them (R. H. S.) and gratefully acknowledge gifts of liver extracts from Glaxo Laboratories Ltd., of vitamin B_6 from Merck and Co., Inc., and of riboflavin from Roche Products, Ltd.

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159. VITAMIN B_1 IN VERTEBRATE MUSCLE

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(*Received 19 August 1940*)

The amounts of vitamin B_1 in the muscular tissues of animals of different species are not the same [Elvehjem & Arnold, 1936; Leong, 1939; Schultz *et al.* 1939]. It has also been found that differences may exist between the vitamin B_1 concentrations in different muscles of the same animal. Although the amount of vitamin B_1 present in muscle depends to some extent on the diet of the animal in question, it seems possible that some of these variations may possess a more fundamental significance. In the present work an attempt has been made to survey the distribution of vitamin B_1 in vertebrate muscles with a view to the elucidation of certain of its functions which are at present somewhat obscure.

Experimental

Method. Throughout the investigation the total vitamin B_1 content of the tissues examined has been determined by the modification of the thiochrome method, details of which have already been published [Pyke, 1939].

The distribution of vitamin B_1 in the muscles of different species of vertebrates

The amount of vitamin B_1 in the muscles of the pig is so greatly in excess of that found in other readily available domestic and experimental animals that a survey was made of as many species as possible in the hope that some other animal might be found whose vitamin stores equalled those of the pig, in which case a clue might be furnished to the function of the high muscular vitamin B_1 level. The animals listed in Table 1 were apparently normal specimens and were killed immediately before analysis.

Table 1. *Distribution of vitamin B_1 in vertebrate skeletal muscle; normal animals*

Name	Muscle	Vitamin B_1 (I.U. per 100 g.)
Pig (*Sus domesticus*)	Various	170–510
Hedgehog (*Erinaceus europaeus*)	Various	59–103
Blotched genet (*Genetta tigrina*)	Leg	95
Goose (*Anser domesticus*)	Various	13–67
Rabbit (*Oryctolagus cuniculus*)	Various	9–63
Kid (*Capra hircus*)	Various	23–63
Fowl (*Gallus domesticus*)	Various	19–57
Rat (*Rattus norvegicus*)	Various	2–35
Sheep (*Ovis aries*)	Leg	34
Ox (*Bos taurus*)	Gluteus maximus	23
Two-wattled cassowary (*Casuarius bicarunculatus*)	Leg	25
West African wood owl (*Strix woodfordii nuchalis*)	—	23
Emu (*Dromiceius hollandiae*)	Leg	15
Bengal vulture (*Pseudogyps bengalensis*)	—	11
Horse (*Equus caballus*)	Various	6
Binturong (*Arctitus binturong*)	Leg	4
Hare (*Lepus europaeus*)	Leg	Nil

Table 2 shows the vitamin B_1 contents of specimens taken from animals dead from the diseases listed in column 3. These and many of the samples in Table 1 were made available by the kind co-operation of the Secretary of the Zoological Society, London.

Table 2. *Distribution of vitamin B_1 in vertebrate skeletal muscle; diseased animals*

Name	Muscle	Cause of death	Vitamin B_1 (I.U. per 100 g.)
Ring-necked parakeet (*Psittacula manillensis*)	—	Impacted cloaca	86
Pileated guan (*Penelope piliata*)	Breast	Enteritis	86
Collared peccary (*Pecari tajacu*)	Various	Pneumonia	38–57
Black-footed penguin (*Spheniscus demersus*)	Breast	Inanition	42
South American flamingo (*Phoenicopterus chilensis*)	—	Old age	38
Lion cub (*Felis leo*)	Leg	Pneumonia	36
Scarlet ibis (*Eudocimus rubra*)	Breast	Endocarditis	30
Grey squirrel (*Sciurus carolinensis*)	Leg	Intestinal obstruction	29
White-tailed mongoose (*Ichneumia albicauda*)	Leg	Endocarditis	19
Black duiker (*Cephalophus niger*)	Leg	Pneumonia	19
Californian sea-lion (*Zalophus californianus*)	Breast	Pneumonia	19
Persian gazelle (*Gazella subgutturosa*)	—	—	17
Crested pelican (*Pelicanus crispus*)	Breast	Peritonitis	15
Crowned pigeon (*Goura cristata*)	Breast	Pneumonia	15
Red brocket (*Mazarma americana*)	Leg	Pneumonia	12
Ocelot (*Felis pardalis*)	Leg	Old age	11
Garnett's bush baby (*Galago garnettii*)	Leg	Rupture of the liver	11
King penguin (*Aptenodytes patagonica*)	Breast	Mycosis	10
Chimpanzee (*Pan satyrus*)	Abdominal	Dysentery	4–10
Rhesus monkey (*Macacus mulatta*)	Various	Tuberculosis	4– 8
Pig-tailed monkey (*Macacus nemestrina*)	Leg	Post-partum haemorrhage	8
Hybrid pheasant (*Tragopan blythi* × *T. satyra*)	Breast	Nephritis	6
Spectacled cayman (*Caiman crocodilus*)	Leg	Tuberculosis	6
Two-spotted palm civet (*Nandinia binotata*)	Leg	Osteomalacia	4
Nylghau (*Boselaphus tragocamelus*)	Leg	Tuberculosis	4
Ard vark (*Orycteropus afer*)	Abdominal	—	4
Silver-grey opossum (*Trichosurus vulpecula*)	Leg	—	4
Wart hog (*Phacochoerus aethiopicus*)	Leg	Carcinoma	2
Crested porcupine (*Hystrix cristata*)	Leg	Gastric ulcer	Nil
Black cayman (*Caiman niger*)	Breast	Pneumonia	Nil
Australian long-nosed crocodile (*Crocodilus johnsonii*)	Tail	Pneumonia	Nil
Southern anaconda (*Eunectes murinus*)	—	Enteritis	Nil

While the random nature of the samples and the pathological condition of the specimens listed in Table 2 make it unjustifiable to draw definite conclusions, it appears that the muscles of the domestic pig are unusually rich in vitamin B_1. The possibility that this superiority of pig muscle is due to the consumption of a diet exceptionally rich in vitamin B_1 must be borne in mind; nevertheless it has been shown by Leong [1939] that no matter how much vitamin B_1 is fed to guinea-pigs, fowls, pigeons or rats, the highest concentration in the muscle is

120 I.U. per 100 g., attained by the pigeon. The maximum figure for the rat reported by Leong was 60 I.U. per 100 g. In the present work a concentration of 35 I.U. per 100 g. only was reached. It seems probable, therefore, that the pig has an innate capacity for retaining vitamin B_1 in its muscles not possessed by other animals.

The variation in vitamin B_1 content of the muscles of random pigs obtained from bacon factories in different parts of the country is shown in Table 3.

Table 3. *Normal variation of vitamin B_1 in pig muscle*

Breed or description	Vitamin B_1 (I.U. per 100 g.)
Imported frozen from Melbourne	170
Berkshire (black)	170
Large white	260
"	260
"	270
"	290
"	290
"	300
"	310
"	340
"	350
"	390
Imported frozen from Queensland	340
Large white × Saddleback	340
Middle white	400
Essex	460
Pig 113 (from Cheshire)	460
Pig 98 (from Cheshire)	510
Pig 91 (from Cheshire)	510

Variation in vitamin B_1 content of different muscles

Up to the present it has merely been shown that even within the variations due probably to differences in diet the vitamin B_1 content of the muscles of the pig is substantially higher than that found in other animals. Differences of a less striking nature also appear between other species. From Table 4, however, it can be seen that vitamin B_1 is by no means evenly distributed between the muscles of the individual animal.

Table 4. *Vitamin B_1 (I.U. per 100 g.) in different muscle groups*

Muscle group	Horse	Kid	Pig
Psoas	6	4	300
Longissimus dorsi	6	15	210
Abdominal muscle	6	34	220
Pectoral muscle	6	38	260
Tongue	6	58	85
Fore leg, ventral	4	30	150
Fore leg, dorsal	6	46	120
Hind leg, ventral	4	23	180
Hind leg, dorsal	6	63	150

When tissues such as muscle contain less than 10 I.U. of vitamin B_1 per 100 g. considerable skill in titration is necessary to obtain accurate figures. In the case of the horse it can, therefore, only be said that its muscles contain a uniformly low level of vitamin B_1. In other animals, however, it is clear that differences exist in respect of individual muscles and a study was therefore made of the

vitamin B_1 content of the psoas, a muscle primarily concerned with movement, and of the longissimus dorsi, the principal function of which is the maintenance of posture. Pig muscles were used.

Comparison of the amounts of vitamin B_1 in psoas and longissimus dorsi of the pig

A litter of 9 Wessex Saddleback pigs was divided into 2 groups at weaning. Group A was fed on a normal commercial meal containing 190 I.U. vitamin B_1 per 100 g. Group B, which was housed under identical conditions, received the same meal with the addition of enough crystalline vitamin B_1 to raise its concentration to 850 I.U. per 100 g. The pigs were slaughtered at $6\frac{1}{2}$ months of age and a cross-section of the carcass was taken at the level of the last rib through (1) outer back fat, (2) inner back fat, (3) longissimus dorsi, (4) psoas, (5) flare fat. In none of the samples from the fatty layers could more than a trace of vitamin B_1, never exceeding 6 I.U. per 100 g., be detected. The figures for the muscle samples are shown below in Table 5. In this table is included, besides the figures for the 2 groups of littermate animals, a third group of figures obtained from swill-fed pigs of similar age which were provided through the kindness of Prof. John Hammond, of the School of Agriculture, Cambridge. While no analysis of the swill was made it almost certainly contained less vitamin B_1 than the cereal meal fed to group A.

Table 5. *Vitamin B_1 content of psoas and longissimus dorsi of pigs fed at different vitamin B_1 levels*

	Longissimus dorsi (I.U. per 100 g.)	Psoas (I.U. per 100 g.)
Swill-fed pigs	160	230
	170	200
Group A (190 I.U. vitamin B_1 per 100 g. food)	285	450
	285	485
	285	400
	315	430
	315	455
Group B (850 I.U. vitamin B_1 per 100 g. food)	315	455
	315	430
	340	485
	285	455

This experiment shows that the amount of vitamin B_1 in the psoas of the pig is consistently greater than that present in longissimus dorsi. Comparing groups A and B, one can conclude that a normal pig ration supplies all the vitamin B_1 that the animal is capable of storing. While the swill-fed group is not strictly comparable, since its members belonged to a different breed, it appears that the reduction in muscle vitamin B_1 is due to deficiency in the diet. However, even at this lower nutritional level, the excess of vitamin B_1 in psoas over that in longissimus dorsi is still maintained.

Vitamin B_1 in cardiac muscle

The vitamin B_1 contents of individual specimens of hearts from animals of different species are listed in Table 6. In general it appears that the concentration of vitamin B_1 in cardiac muscle is higher than that found in skeletal muscle. Although variations exist from one species to another, there is some degree of uniformity in the vitamin B_1 levels found.

Table 6. *Vitamin B_1 in heart muscle* (I.U. *per 100 g.*)

Kid	170
Hen	150
Rabbit	150
Pig	140
Hedgehog	130
Horse	80–130
Sheep	110
Rat	100
Goose	70

When cardiac muscle, however, is examined in greater detail, it is found that the vitamin B_1 is not uniformly distributed throughout the heart. Figures are given in Table 7 from which it appears that in the two species examined the concentration of vitamin B_1 is higher in ventricular muscle than in auricular.

Table 7. *Vitamin B_1 distribution within the heart* (I.U. *per 100 g.*)

	Pig	Horse
Auricles, right	125	34
left	115	40
Ventricles, right	170	74
left	200	80

Vitamin B_1 in red and white muscle

There is some evidence that red muscle contains more vitamin B_1 than white. For example, the psoas of the pig is a darker red than the longissimus dorsi and contains more vitamin. In the case of the rabbit, it is difficult at all times to measure variation between muscles since in many cases the absolute level of vitamin B_1 may be very low. Results from 3 animals, however, are shown in Table 8.

Table 8. *Vitamin B_1* (I.U. *per 100 g.*) *in the red and white muscles of the rabbit*

	Red muscles		White muscles	
Rabbit 1	Soleus (right leg)	55	Vasta lateralis (right leg)	32
	Soleus (left leg)	49	Vasta lateralis (left leg)	36
	Pectineus (pooled)	63	Gluteus max. (right)	30
			Gluteus max. (left)	34
Rabbit 2	Soleus, crureus (pooled)	32	Gastrocnemius	29
	Pectineus	42	Longissimus dorsi	29
Rabbit 3	Soleus	12	Vasta lateralis	10
	Pectineus	28	Gracilis	10

The state of vitamin B_1 in muscle

If muscle is minced, shaken vigorously with water or pepsin-HCl solution and samples are immediately taken for the thiochrome test, figures are obtained which give the amount of free vitamin B_1. The remainder of the sample can then be digested with the pepsin-HCl solution and a second aliquot removed for test. This second figure has been tentatively assumed to be free plus "residual" vitamin B_1, while the final result after an additional digestion with takadiastase has been assumed to comprise free plus "residual" vitamin B_1 plus cocarboxylase. Cocarboxylase may break down after the death of the muscle and although it does not appear to do so in the acid pepsin solution it is possible that the second determination made after 16 hr. digestion in pepsin-HCl includes a proportion of the cocarboxylase originally present. The following experiments must, therefore, be accepted with this possibility of error in mind.

A sample of pig psoas examined within a few minutes of the death of the animal was found to contain a total of 455 I.U. per 100 g. of vitamin B_1. Of this total, 11 I.U., or 2·4 %, were free vitamin B_1. Cocarboxylase, calculated as vitamin B_1, amounted to 170 I.U., or 37 %. Aliquots of the minced muscle were allowed to stand at room temperature, protected from evaporation, and were analysed periodically. The results are shown in Fig. 1, from which it can be seen that cocarboxylase breaks down to free vitamin B_1. Ochoa [1939] has demonstrated that tissues contain an enzyme capable of phosphorylating vitamin B_1. This enzyme may also catalyse the reverse reaction and thus break down cocarboxylase. The fact that a proportion of the cocarboxylase in the present experiment still remained in pig muscle 24 hr. after death is in agreement with the finding of Westenbrink & Dorp [1940] that the enzyme is inhibited by the accumulation of free vitamin B_1.

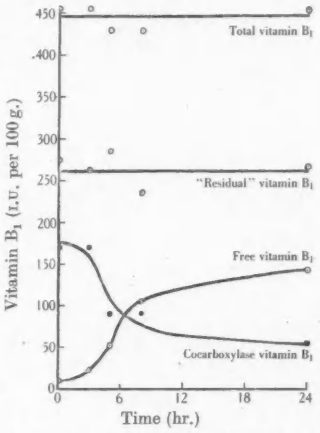

Fig. 1. Breakdown of cocarboxylase in minced pig psoas allowed to stand at room temperature.

The breakdown of cocarboxylase in this manner during the autolysis of muscle does not appear to be universal in all muscular tissues. The results of an experiment on horse's heart muscle are shown in Table 9.

Table 9. *Partition of vitamin B_1 compounds during autolysis of horse's heart* (I.U. *per 100 g.*)

Time after death hr.	Free vitamin B_1	Cocarboxylase	Residual vitamin B_1	Total vitamin B_1
1·5	4	63	13	80
4·5	4	55	21	80
24	8	23	55	86

From a suggestion of Ochoa [Banga *et al.* 1939] it seems probable that "residual vitamin B_1" is in fact vitamin B_1-monophosphate. If this is the case, autolysis of heart muscle of the horse results in the breakdown of cocarboxylase to form the monophosphate, whereas in pig psoas cocarboxylase is completely dephosphorylated to free vitamin B_1. In the pig's muscle, however, the phosphate group is apparently not removed from the "residual" or monophosphate-vitamin B_1.

Discussion

The results which have been presented in this paper raise possibilities of considerable interest. A hypothesis which might be worthy of further investigation, is that vitamin B_1 in muscle is specially concerned with the glycogen cycle. The muscles of the horse, which are consistently low in vitamin B_1, contain high percentages of glycogen even after prolonged exercise [Mathews, 1939], whereas pig muscles, which are exceptionally rich in vitamin B_1, contain small amounts of glycogen which are rapidly depleted by exercise [Bate Smith, 1937]. Again, it has been shown that the psoas muscle of the pig contains more

vitamin B_1 than longissimus dorsi. In agreement with this result, Callow [1938] has deduced by his technique of measuring the "ultimate *p*H" that in practically all of 30 pigs there was less glycogen in the psoas than in longissimus dorsi.

The results of the experiments on cardiac muscles and on red and white rabbit muscles supply additional evidence. It appears that there is more vitamin B_1 in the ventricles than in the auricles of the hearts of pig and horse. If vitamin B_1 is inversely related to the glycogen level one would expect to find less glycogen in the ventricles than in the auricles, and, according to Cruikshank [1936], this is the case. The work on red and white muscles, if it can be confirmed, is also interesting. It appears that the red muscles of rabbits contain more vitamin B_1 than white muscles. Noll & Becker [1936] have shown that red rabbit muscle only contains 65–80 % as much glycogen as white muscle. Red muscle contains more sarcoplasm than white and the granules of glycogen are found in the sarcoplasm and not in the fibrils. It seems possible, therefore, that vitamin B_1 is also present in the sarcoplasm.

Williams & Spies [1938] have reviewed the literature on the present knowledge of the relationship between vitamin B_1 and glycogen. Much of it is inconclusive but certain experiments with pigeons show that deficiency of vitamin B_1 raises the level of glycogen in the liver.

Summary

1. The vitamin B_1 contents of the muscles of a number of animals of different species have been determined. The muscles of the pig appear to be several times richer in vitamin B_1 than those of any other animal.

2. Variations occur in the amount of vitamin B_1 in different muscles in the animal body. In the pig there is more vitamin B_1 in psoas than in longissimus dorsi.

3. Heart muscle contains more vitamin B_1 than is normally found in skeletal muscle. There is a higher concentration of vitamin B_1 in the ventricles than in the auricles.

4. Red muscles of rabbits appear to contain more vitamin B_1 than white muscles.

5. If muscle is allowed to stand after death cocarboxylase breaks down and free vitamin B_1 appears in pig's muscle, while vitamin B_1-monophosphate is probably formed in horse's heart.

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160. THE ESTIMATION OF VITAMIN B_1 IN URINE

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(*Received 30 September 1940*)

VITAMIN B_1 can be estimated by measuring the intense blue-violet fluorescence of its oxidation product thiochrome [Jansen, 1936]. When applied to urine the thiochrome method gives results of the same order as the bradycardia method [Karrer, 1937; Wang & Harris, 1939] and the colorimetric method of Melnick & Field [1939, 1, 2], but interfering substances in urine have made quantitative estimations difficult. Urine gives a "blank" fluorescence which is reduced to an unknown extent by the ferricyanide employed to oxidize the vitamin to thiochrome. The recovery of thiochrome from vitamin B_1 added to urine is incomplete and variable.

In the method described here the synthetic zeolite used as an adsorbent for the vitamin has more specific properties than the activated earths formerly employed. The "blank" fluorescence is much less than in other methods, and the recovery of thiochrome, although incomplete, is constant under suitable conditions. The method, which is simple and rapid, has been applied only to human urine.

A study of the rate of urinary excretion of ingested vitamin B_1 has given information about controlling factors, and the minimum period of urine collection required in tests of the nutritional level has been estimated.

Methods

The technique has already been outlined [Jowett, 1939]. It is simpler than that of Hills [1939], which was published later. The adsorbent used is Decalso, a product of the Permutit Co., Ltd. Fluorescence is measured with the Pulfrich photometer.

Treatment of urine. 24 hr. specimens are collected in bottles containing 5 ml. glacial acetic acid. Urine so acidified (of *p*H 3·4–4·8) may be kept at room temperature for several days, and in the refrigerator for several weeks, without loss of vitamin B_1. For shorter periods of collection proportionately less acetic acid is added, but the resulting *p*H is then sometimes higher and requires adjustment to about 4·5. If a heavy precipitate is present the urine is warmed before it is sampled. Urine need not be filtered before analysis unless blood is present, in which case cell-pigments must be removed.

Reagents. Vitamin B_1 solution, prepared by diluting the contents of a "Benerva" (Roche) ampoule to 10 μg./ml. with a buffer solution of *p*H 4 (0·03 *M* acetic acid + 0·0075 *M* acetate), maintains its strength in a refrigerator for at least 3 months. *iso*Butanol (B.D.H. Laboratory Reagent) may be used as received, but gives a lower "blank" when redistilled, as stated by Wang & Harris [1939]. The pure solvent may be recovered after the analysis by fractionation of the neutralized wet alcohol mixture. Powdered Decalso is prepared from the dry crystalline material by grinding it to a fine powder, which is suspended in water and decanted several times to leave coarse particles behind. Fine

particles are removed by rejecting the supernatant after very brief centrifuging. The precipitate is washed on the centrifuge with alcohol and acetone, dried roughly at 60–80°, then completely in a desiccator evacuated with a water-pump, and finally powdered gently and kept in a desiccator.

Analytical procedure. It is convenient to analyse several urines at the same time. In each analysis 3 equal amounts of urine are taken, one for the "blank", a second for the "test" and a third for estimating vitamin recovery. The third specimen may be omitted for some of the urines if the conditions give uniform recovery. A "reagent blank", omitting urine, may also be required.

1–20 ml. of acidified urine, containing preferably 1–3 μg. vitamin B_1, are measured into a centrifuge bottle containing 1·5 ml. 0·2*N* acetic acid, the purpose of which is to buffer the Decalso. To the third bottle in each analysis are added 6 μg. vitamin B_1. Each bottle is filled to the shoulder with water, the total volume being about 23 ml., after which 150 ($\pm$7) mg. powdered Decalso are introduced and shaken into suspension. Centrifuging for a suitable short period packs the Decalso lightly. The supernatant (the *p*H of which may require determination with B.D.H. "4·5" Indicator) is poured off carefully and the bottles are inverted to drain for a few minutes. The neck of each bottle is wiped, 1·5 ml. methanol are added and the Decalso is shaken loose from the bottom.

The following procedure is then applied to each bottle in turn, "blanks" being treated first, then "tests" and finally "vitamin recovery" bottles. The Decalso is shaken into suspension and a stream of N_2 is passed through the liquid. 1 ml. 5·6*N* NaOH is added, followed by 2 ml. 0·75% $K_3Fe(CN)_6$ (or by 2 ml. water for a "blank"). 10 ml. *iso*butanol are added and the mixture is stirred with N_2 for 15–30 sec. longer. The procedure takes about 1½ min. Finally 9·5–10 ml. of the supernatant alcoholic layer are pipetted into a test tube containing 0·5 ml. ethanol and shaken. Ethanol clears the slightly turbid fluid.

Measurement of fluorescence. Fluorescence is measured with the Zeiss-Pulfrich photometer by the substitution method through an L III filter, which transmits blue and violet light. The fluorescent solution, in an uncovered rectangular cell 3 cm. long, is matched against a piece of Ilford Fluorazure Screen mounted at 45° from the vertical, the fluorescence of which is reduced by filter paper pressed tightly against it with glass. Cell and screen are illuminated from above by ultraviolet light, filtered through Wood's glass, derived from a Hanovia Universal Fluorescence Lamp, the position of which is roughly reproducible. The use of vertical illumination eliminates special focussing and filters.

Readings can be made within 5 min. of entering the dark room. If eye-fatigue is minimized by rapid matching of not more than 20 solutions on each occasion, an accuracy of 1–5% is obtained after some practice. Matching is best at moderate intensities. Fluorescence is expressed on an arbitrary scale as the ratio of the drum-readings multiplied by 100. Each analysis or set of analyses is calibrated by the inclusion of specimens with added vitamin. After subtraction of the appropriate "blank", fluorescence is proportional to thiochrome concentration over a range extending at least to that derived from 25 μg. of adsorbed vitamin.

Discussion of the method

Decalso in the convenient powder form has hitherto not been used for purifying vitamin B_1, though American workers have used it in a column. Other cations in urine compete with vitamin B_1 for adsorption, and only under restricted conditions is adsorption of the vitamin complete. Melnick & Field [1939, 2], whose less sensitive method requires about 200–400 ml. urine, had to

resort to a troublesome extraction before the adsorption. In the present method direct treatment of urine is satisfactory if the *p*H is correct and the ratio of urine volume to weight of Decalso is not too high. The optimum *p*H under the analytical conditions is about 4·5 and the yield is lowered by about 10% at *p*H values of 3·9 and 5·1. The combined buffering action of acetic acid and Decalso, which takes up hydrogen ion, usually leads to a *p*H very close to 4·5 without special adjustment of the *p*H of the acidified urine. Since the salt content of urine is very variable no definite upper limit can be set to the amount of urine from which the vitamin is completely adsorbed. Adsorption is 95–100% complete when the urine volume is equivalent to 1/300 of a 24 hr. specimen (about 3–8 ml.), but doubling this amount of urine may sometimes lead to less complete adsorption. It is not essential that adsorption should be complete, since the recovery can be estimated in each analysis, but if the recovery is much lowered the accuracy is reduced.

Recovery. The thiochrome derived from adsorbed vitamin by the combined processes of elution, oxidation and extraction described is only about 68% of that derived from the vitamin in solution treated in the same manner in the absence of Decalso. The incomplete recovery is apparently due to incomplete elution of the vitamin or to its destruction during elution. In founding a quantitative method on an incomplete recovery, the recovery should be consistent, even though it can be estimated in each analysis. Investigation has shown that the recovery varies little either when different batches of reagents are used or when the analytical conditions are varied far beyond the limit of accidental variation of technique. The recovery from pure solutions of vitamin is about 68% over a wide range of concentration. The recovery from a large number of urines under the conditions described (about 95% adsorption) averages 65%, with a standard deviation about ± 3. Definite alteration of recovery has been observed only when too much urine is taken or in analysing unfiltered specimens of menstrual urine containing blood. The very low recovery in presence of blood, which is due to destruction of vitamin B_1 by haematin in an alkaline medium [cf. Schroeder, 1939], was found to rise to normal when the clotted red cells were filtered off before analysis.

Other thiochrome methods give much less consistent recoveries as far as details are available. Westenbrink & Goudsmit [1937] obtained varying yields up to 70%, and according to Hills [1939] the method of Wang & Harris [1939] gives similar recoveries. Hills [1939] obtained an average yield of 76% with a standard deviation of ± 17, the great variability being due to interfering substances in urine, for increasing the amount of adsorbent lowered the yield. In the present method excess of Decalso does not lower the yield. It is possible that the use of Decalso in a column, without previous extraction of urine but with separate procedures for elution and oxidation of the vitamin, may give a high and consistent yield of thiochrome at the expense of more labour. In the only work on such lines [Hennessy & Cerecedo, 1939] urine is analysed only incidentally and the method cannot be regarded as established for urine.

The "blank" fluorescence. In the absence of ferricyanide, fluorescent substances in urine give rise to the appearance in the *iso*butanol solution of a fluorescence which has been regarded as a "blank" to be subtracted from the "test" fluorescence. Hills [1939] has however found evidence that ferricyanide reduces to an unknown extent the green fluorescence arising from urine constituents. The uncertainty introduced into the method of Hills is considerable, for the amount of vitamin estimated is less than 4 μg. and the "blank" fluorescence expressed in terms of vitamin is 1–2 μg. when the usual amount of about

9 ml. urine is analysed, or about 0·6 μg. with 2 ml. urine. The methods of Westenbrink & Goudsmit [1937] and of Wang & Harris [1939] give "blanks" of the same order.

The present method gives much lower "blanks", and it becomes worth while to distinguish between a "reagent blank" (determined in absence of urine) and the observed "total blank", the difference being called the "urine blank". In terms of vitamin recovered from urine, the "reagent blank" with redistilled *iso*butanol is about 0·09 μg. and is unaffected by ferricyanide. The "urine blank" with amounts of urine of the order of 1/300 of the 24 hr. output averages 0·1–0·15 μg. In 800 analyses only 2 or 3 "urine blanks" as high as 0·4 μg. have been found. The "total blank" fluorescence is usually blue in colour.

The "urine blank" is still high enough to introduce uncertainty into the determination of low vitamin concentrations. If from the "test" reading the "reagent blank" is subtracted instead of the "total blank", the calculated 24 hr. output of vitamin averages about 35 μg. higher (the corresponding figure in Hills's method being about 7 times as great).

An attempt has been made in former methods to eliminate the "blank" difficulty by the use of a supposedly optimum amount of ferricyanide. It has been found by Hills with Clarit, and confirmed in this laboratory for Decalso adsorbates from urines of very low vitamin content, that although a certain amount of ferricyanide may give a maximum "test" reading, this amount is insufficient to give full thiochrome production from added vitamin. The supposedly optimum amount has to be determined or guessed. There are also the objections (1) that destruction of non-thiochrome fluorescence, though lessened, has not been shown to be eliminated, (2) that the amount of ferricyanide required to give maximum "test" readings will depend on the ratio of vitamin to fluorescent substances in the sample of urine and (3) that the conversion of added vitamin into thiochrome will be more variable and more liable to technical error.

In the present method a considerable excess of ferricyanide is used. About 0·1–0·2 ml. 0·75 % $K_3Fe(CN)_6$ gives the maximum fluorescence with urines of low vitamin content, while about 0·2–0·4 ml. lowers this fluorescence and raises the yield of thiochrome from added vitamin to levels which are not altered by amounts up to 2 ml.

Since the "urine blank" is usually small it is difficult to investigate, but several rough tests agree in suggesting that the excess of ferricyanide approximately halves the "urine blank".

(1) Sulphite destroys vitamin B_1 more rapidly than the substances responsible for the "urine blank", e.g. in one experiment 0·02 *M* sulphite at 100° and *p*H 4·9 destroyed half the vitamin every 1½ min., while the "urine blank" decreased only 20 % in 14 min. With urine freed from vitamin by sulphite the effect of ferricyanide on the residual "urine blank" can be determined.

(2) The "urine blank" does not usually rise in proportion to the amount of urine analysed. By analysing several different amounts of urine of high "blank" and low vitamin content, a rough estimate can be made of what proportion of the "urine blank" applied as a correction gives the most consistent estimate of vitamin content.

(3) If the "total blank" is subtracted, urines of low vitamin content very occasionally give a negative vitamin value which becomes positive if the correction for the "urine blank" is halved.

The most suitable "blank" to subtract is therefore taken to be midway between the "total blank" and "reagent blank". These two values represent

the limits of choice of "blank". The error involved in the mid-choice cannot on the average exceed ± 18 μg. for a 24 hr. urine, and it is probably less. The calculation from the fluorescence readings is illustrated by the following example: "reagent blank" 0·7, "total blank" 1·9, "test" 12·3, "test+6 μg. vitamin" 73·5. The amount of vitamin is $6\ (12{\cdot}3-\frac{1}{2}\ \{0{\cdot}7+1{\cdot}9\})/(73{\cdot}5-12{\cdot}3)$, or 1·08 μg.

Application of the method. According to Robinson *et al.* [1940], men on a diet low in vitamin B_1 excrete 0–90 μg. daily in their urine. The present method has a "blank" error that may possibly average as high as 18 μg. per 24 hr. and a photometric error (for low values) of about 5 μg. per 24 hr., and it therefore does not measure such excretions accurately. The dietary excretion is however only of limited value in assessing the nutritional level. According to Robinson *et al.* [1940] urine must be collected for 24 hr. while the subject is on a diet of adequate vitamin content. The method does not apply in achlorhydria or during alkaline medication, it entails delay before therapy, and it cannot be used to follow the progress of therapy.

The method of determining urinary excretion after an oral dose of vitamin has fewer limitations. With a small dose, such as the 1 mg. recommended by Hills [1939], the excretion depends appreciably on the current vitamin intake. A 5 mg. dose, however, leads to a vitamin excretion which is much less influenced by current dietary or recent therapeutic intake and is also large enough to possess therapeutic value. The present analytical method is well suited for determining the excretion following a 5 mg. dose, which for a man adequately supplied with the vitamin amounts to 400–1200 μg. in 24 hr. The uncertainty due to the "blank" becomes unimportant and the standard error of a single estimation is about 7%, due mainly to photometric error. Since the range of normal response and the variability of abnormal response are great, this accuracy is quite satisfactory. With care and duplication more accurate values may be obtained for special purposes.

The rate of urinary excretion of vitamin B_1

The urinary response to an oral dose of 5 mg. of vitamin B_1 can be estimated by measuring the excretion during the following 24 hr. Experiments reported here indicate that shorter periods of urine collection will serve. They also cast light on the part played by the stomach and kidney in absorption and excretion of the vitamin.

Four adult male laboratory workers ingested 5 mg. of the vitamin immediately after breakfast. While the excretion in the first $2\frac{1}{2}$ hr. was variable, the proportion of the 24 hr. excretion of vitamin which took place in the first 5 hr. was fairly constant at about 68% (Table 1). Correction can be made for the excretion of vitamin of dietary origin, assuming this to be the same as on the previous day and to be spread evenly over the 24 hr. The 5 hr. excretion of vitamin derived from the test-dose is then found to be about 75% of the 24 hr. figure.

Table 1. *Excretion of vitamin $2\frac{1}{2}$, 5 and 24 hr. after ingestion*

Subject	μg./24 hr. on normal diet	μg./24 hr. after test dose	% of 24 hr. excretion after test dose which is excreted in	
			$2\frac{1}{2}$ hr.	5 hr.
C.	39	652	35	65·5
D.	80	768	22	69
J.	144	1002	41	69
R.	121	704	—	70

In an otherwise similar experiment with 8 mental patients the 5 hr. excretion was determined on a different day from the 24 hr. excretion; the former averaged 71 % of the latter, but there was greater variation among the individuals. In one case the percentage was only 27, but the 5 hr. urine volume was only 90 ml., which suggests that retention of urine in the bladder can lead to low results in a 5 hr. test.

Table 2. *Excretion at short intervals after a dose*

Exp. 1 (day 1)		Exp. 2 (day 3)		Exp. 3 (day 13)	
Time hr.	μg. vitamin B_1	Time hr.	μg. vitamin B_1	Time hr.	μg. vitamin B_1
0– 1	28	0 – 0·8	7	0 – 0·5	6
1– 2	151	0·8– 1·5	47	0·5– 1·6	99
2– 3	137	1·5– 2·5	129	1·6– 2·4	50
3– 4	43	2·5– 3·1	134	2·4– 3·1	37
4– 5	38	3·1– 3·6	57	3·1– 4·0	45
5– 6	31	3·6– 5·0	98	4·0– 5·0	18
6– 7	19	5·0– 6·6	61	5·0– 8·0	74
7– 8	21	6·6– 8·0	43	8·0–23·0	182
8–23	187	8·0–24·0	226		
Total excretion					
(*a*) By addition of above figures	655		802		511
(*b*) By analysis of pooled aliquots	735		789		518

The excretion was followed at much shorter intervals with subject J. (Table 2). In Exps. 1 and 2, 5 mg. vitamin B_1 were ingested immediately after breakfast, in Exp. 3, 0·8 hr. before breakfast. Times were reckoned as before from the time of ingestion of the vitamin. The estimated rates of vitamin excretion are plotted in Fig. 1, the times at which the maxima occur being well defined although the heights of the maxima are only approximate. It is clear (Exps. 1 and 2) that the time of the maximum can vary from day to day in the same individual. The rate of excretion falls greatly 2 hr. after the maximum. The maximum occurs earlier when the vitamin is ingested before the meal (Exp. 3), but the amount of vitamin excreted is less. This result agrees with those of Melnick *et al.* [1939], who followed the excretion at 4 hr. intervals when a 5 mg. dose had been ingested either 3 hr. before breakfast or immediately after a heavy meal. These authors suggest that much of the vitamin is destroyed before absorption when taken into a fasting stomach.

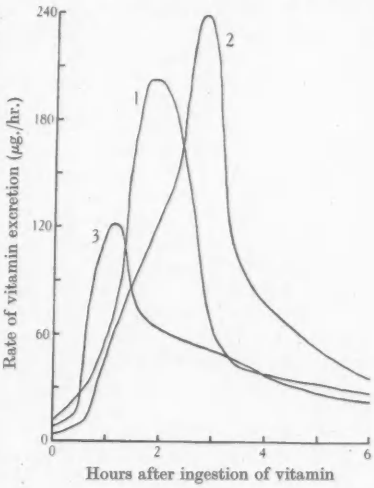

Fig. 1.

It is probable that the site of absorption of vitamin B_1 is the small intestine, and injection experiments have shown that once the vitamin reaches the circulation excretion takes place rapidly. If the vitamin is absorbed rapidly from the intestine, then the time-interval between ingestion and maximum excretion of the vitamin will depend mainly on the rate of emptying of the stomach. The rates of excretion shown in Fig. 1 support the view that maximum excretion occurs soon after the passage of most of the vitamin into the intestine, and that the variability of the lag between ingestion and maximum excretion is due to variability in the rate of emptying of the stomach.

The vitamin excretion after ingested doses has already been followed at hourly intervals by Westenbrink & Goudsmit [1938], but the time-relation to meals was not indicated. Their finding that most of the excretion of the dose took place in 3 hr. is therefore not an adequate basis for the method adopted by Hills [1939] of determining the excretion during 3 hr. after ingestion of a test-dose with breakfast.

The present experiments indicate that, when a test-dose is ingested with breakfast, urine should be collected for at least 5 hr. to obtain a fairly uniform proportion of the resulting excretion of vitamin. If there is a likelihood of delayed stomach or bladder function, the period should be extended. The data of Melnick *et al.* [1939] make it probable that a period of 8 hr. is required after ingestion of the dose with a heavy meal.

How the kidney deals with vitamin B_1 does not appear to have been discussed. We are not concerned with the phosphorylated vitamin, which is not found in urine or (apparently) plasma. If the diet is adequate the concentration of the free vitamin in daytime urine is of the order 0·04–0·16 μg./ml. According to analyses by thiochrome methods the concentration in plasma is of the same order, Ritsert [1939] finding 0·03–0·15 μg./ml. in blood and Hennessy & Cerecedo [1939] 0·09–0·12 μg./ml. On the other hand Goodhart & Sinclair [1940], relying on a small difference between total and phosphorylated vitamin determined by different methods, estimate the free vitamin in blood to be 0·01 μg./ml., which is less than in urine. These discordant results do not show whether the kidney concentrates the vitamin from plasma.

A definite decision on this question can be arrived at from the experiments summarized in Table 2. In Exp. 2 during the period 1·5–3·1 hr. after ingestion of 5 mg. of vitamin the average concentration in the urine exceeded 3 μg./ml. If the whole of the 5 mg. dose were present in the blood plasma the plasma concentration would be only 1·5 μg./ml. But vitamin B_1 leaves the circulation rapidly; from the blood analyses of Ritsert [1939] it can be deduced that vitamin injected intravenously into rabbits is distributed as if throughout the body water in about 10 min. Clearly only a small proportion of an ingested dose can be present in the plasma for a period of, say, an hour. The conclusion to be drawn is that the kidney can concentrate vitamin B_1 from plasma to a marked degree, perhaps 20 times or more.

Summary

1. A rapid and simple method of estimating vitamin B_1 as thiochrome is applied to urine. The powdered Decalso used is a more specific adsorbent of the vitamin than are activated earths, and gives lower "blanks" and more consistent yields. Fluorescence is measured with the Pulfrich photometer.

2. The method does not estimate low dietary excretions accurately, but is suitable for measuring the response to test-doses, such response being the best test of nutritional level.

3. Measurements of urinary excretion at short intervals after ingestion of 5 mg. of vitamin show that the time-lag before excretion depends mainly on the time required for the stomach to empty, and that the shortest suitable period of urine collection in saturation tests is 5 hr. The kidney can concentrate vitamin B_1 from plasma.

The author is indebted to Roche Products, Ltd., for supplies of vitamin B_1, and to the Permutit Co., Ltd., for Decalso. He is grateful to Dr W. M. Ford Robertson for his interest in this work, and to other colleagues for their co-operation.

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161. THE ANALYSIS OF HAIR KERATIN

1. A METHOD FOR THE QUANTITATIVE REMOVAL OF CYSTINE FROM KERATIN HYDROLYSATES

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(*Received 12 September 1940*)

The amount of accurate information concerning the composition of keratin hydrolysates is very limited. Wool has received more study than most keratins, but even in it about 60% of the protein has still to be accounted for. More knowledge about the amino-acids in keratin hydrolysates should not only prove useful to those interested in the constitution of such proteins but might also have the immediate practical result of revealing a new source for some amino-acids.

It is a curious fact that in spite of the availability of human hair, its chemical composition has never been thoroughly studied. While numerous papers appeared between 1880 and 1920 dealing with this keratin, most of them were concerned with the sulphur and cystine contents and with the effect of age, sex, race etc., on the sulphur content of hair; most of these data are based on unreliable methods. Recent studies have led to the development of more trustworthy procedures [Rimington, 1929] and the results so obtained have invalidated previous conclusions.

Buchtala [1907] attempted to apply Fischer's ester-distillation method to a hair hydrolysate. The mono-amino-acids thus obtained accounted for only about 30% of the protein and the analytical data for some of the acids isolated did not indicate a very high degree of purity; only limited quantitative significance can be attached to the figures given (glycine 9·1, alanine 6·9, leucine 12·1, phenylalanine 0·6, tyrosine 3·3%). Buchtala also reported values of 8·0% for glutamic acid and 14·5% for cystine, the latter being calculated from the sulphur content of impure fractions obtained by direct crystallization. Block *et al.* [1939] have reported more recent determinations of some of the amino-acids in human hair, but the summation of these latest data accounts for less than 40% of the hair keratin.

The instability of cystine and its derivatives has been found to lead to difficulties in the analysis of keratins no matter which of the usual methods for the separation of the amino-acids is used; if a procedure could be devised for the complete removal of cystine, without at the same time rendering the solution of the remaining amino-acids unworkable, the problem of keratin analysis would be greatly simplified. Such a method will be described in this paper.

Older procedures for removing cystine from keratin (hair or wool) hydrolysates involved neutralization of the acid used for the hydrolysis by alkali or buffering with sodium acetate, thereby producing large quantities of inorganic salts which remained with the other amino-acids. Not only do such methods introduce much undesirable inorganic matter, but they usually leave one-third

to one-half of the cystine in solution in the mother liquors [Barritt, 1927; Wilson & Lewis, 1927; Toennies & Bennett, 1935–6]. Separation of the basic amino-acids from such a mixture is difficult [Vickery & Leavenworth, 1929] and of the other amino-acids almost impossible. If sulphuric acid be used for the hydrolysis it may be removed with baryta, but there are several disadvantages to this procedure. Addition of powdered baryta or of hot saturated solutions is liable to cause excessive local alkalinity with partial racemization and destruction of cystine; cold saturated baryta solution cannot conveniently be used because of the excessive volume required. In both cases large losses of nitrogen occur by adsorption on the precipitate of $BaSO_4$.

A few years ago it occurred to one of us (C. C. L.) that by some modification of the cuprous oxide procedure [Hopkins, 1929; Pirie, 1931] which proved so useful in the hands of Vickery & White [1933] and others, cystine might be removed from keratin hydrolysates without spoiling the mixture for subsequent investigation. It was hoped that cuprous oxide might be used (1) to reduce the cystine quantitatively to cysteine, thus avoiding the introduction of zinc or tin, (2) to precipitate the cysteine as cuprous mercaptide, (3) to neutralize excess (mineral) acid to such a *p*H as to give quantitative removal of the mercaptide, thus avoiding the addition of alkali or acetate. Preliminary experiments in 1934–5 showed that cuprous oxide would indeed reduce cystine to cysteine and precipitate it under certain conditions, but the observation was not followed up at the time and shortly afterwards Rossouw & Wilken-Jorden [1935] reported a similar finding, viz. that cuprous chloride would reduce cystine and precipitate cysteine cuprous mercaptide. Owing to the pressure of other duties the matter was not touched again until 1937 when the possibility came to mind that, by using a sufficiently concentrated hydrolysate, the cuprous oxide (or chloride formed by the residual HCl) would be dissolved by the amino-acids, allowing the reactions to occur in solution without the necessity of using a salt or acid solvent for the cuprous compound as was done by Rossouw & Wilken-Jorden.

Conditions for the realization of all four of these hopes were soon established. A simple procedure was developed which not only removes cystine from keratin and other hydrolysates in such a way as to leave the mother liquor in a workable condition but which also permits a good recovery of pure, crystalline cystine. This procedure has been used in this laboratory for several years and since it has proved of great value to us for certain types of investigation it may be useful to others.

Two equations have been proposed to explain the appearance of cysteine when heavy metals are used to precipitate cystine:

$$3\,RSSR + 3H_2O \rightarrow R.SO_2H + 5RSH, \qquad \text{.....(1)}$$

$$2RSSR + 2H_2O \rightarrow R.SO_2H + 3RSH. \qquad \text{.....(2)}$$

However, in the presence of Cu_2O the reaction may follow the course indicated by equations (3) and (4) which may be summed to give equation (5):

$$RSSR + Cu_2O + H_2O \rightarrow 2RSH + 2CuO \qquad \text{.....(3)}$$

$$2RSH + Cu_2O \rightarrow 2RSCu + H_2O \qquad \text{.....(4)}$$

.....

$$RSSR + 2Cu_2O \rightarrow 2RSCu + 2CuO. \qquad \text{.....(5)}$$

In order to discover whether equation (1), (2) or (5) represents the mechanism, an attempt was made to recover the cysteine quantitatively. The maximum yield possible, according to equation (1) would be 83·3 %, according to equation (2) 75 % and from (3) 100 %. The recovery in pure crystalline form of 90 % of

the amount of cystine taken, as accomplished in the work described below, eliminates (1) and (2) and strongly supports equation (5).

The use of cuprous oxide for the direct removal of cystine from a protein hydrolysate was studied by adding known amounts of cystine to solutions of pure amino-acids and to hydrolysed gelatin. When the removal and recovery of cystine from these mixtures were shown to be satisfactory, hydrolysates of several types of hair were treated by the procedure adopted; cystine was quantitatively removed (and readily isolated in good yield) leaving the other amino-acids in a form easily amenable to further examination. From many preliminary experiments four only will be described in detail and one separation of cystine from a human hair hydrolysate will be outlined.

Experimental

Exp. 1. Precipitation of cystine added to a gelatin hydrolysate

The first experiment showed that complete removal of cystine from a complex amino-acid mixture could be easily accomplished, without preliminary reduction, by direct addition of cuprous oxide to the hydrolysate.

A known amount of cystine (2·5 g.) was dissolved in *N* HCl and added to 125 ml. of a melanin-free gelatin hydrolysate, 20 % in concentration with respect to amino-acids, from which much of the excess HCl had already been removed. After concentration *in vacuo* to a thick sludge the mixture was diluted to exactly 100 ml. and aliquots of 20 ml. (equivalent to 0·5 g. cystine in about 5·5 g. total amino-acids) were pipetted into a series of mortars. Different quantities of red cuprous oxide (1, 2, 3, 5 and 10 g. respectively) were added to the mixtures. With persistent grinding the oxide dissolved slowly to form a dark greenish-brown solution. In the last two mixtures there was an excess of the red oxide and in them a white turbidity formed. Each of these mixtures was diluted by pouring them separately into 4 vol. of ice-cold water; heavy white precipitates (A) formed at once. These were removed by centrifuging; an equal volume of cold water was added to each of the supernatants and more white precipitate (B) appeared, which was similarly removed. The bluish-green supernatants (volume now 200 ml.) were poured into 200 ml. cold water and left in the refrigerator for several hours. A further small white precipitate (C) appeared.

The use of various volumes of water for the precipitation showed that with less than 10–12 vol. the immediate precipitation was followed by a slower one which required many hours for completion, but which could be accelerated by further dilution. The dilution finally chosen as giving the maximum rate and completeness of precipitation, without excessive dilution, was 15 vol. of water.

The several precipitates (A, B and C) from each mixture were combined, washed twice with cold water, suspended in water and freed from copper with H_2S. The several blue-green supernatants were also freed separately from copper with H_2S. After careful removal of H_2S the supernatants were tested for cystine with sodium nitroprusside in the presence of sodium cyanide. No cystine could be detected in those supernatants which had had 0·4 g. Cu_2O or more per g. of total amino-acids, i.e. the amount of cystine left unprecipitated in a mixture such as the above was less than 4 mg. (0·01 mg. per ml., which is about the limit of sensitivity of this test). The solutions obtained by removing the copper from the white mercaptide precipitates were found (iodimetrically) to contain most of the cystine, the greater part being in the form of cysteine. However, more nitrogen was found in these solutions than could be accounted for as cysteine [cf. Graff *et al.* 1937]. Since some cupric ion is formed during the

reduction of the cystine, part at least of those amino-acids forming insoluble cupric salts (leucine, phenylalanine, tyrosine, aspartic acid) would probably also be precipitated under these conditions. The removal of these by a suitable washing technique was next studied.

Exp. 2. Washing of the cuprous mercaptide precipitate

Some preliminary tests of the washing of the crude cuprous mercaptide precipitate showed that water alone dissolved the "non-cystine-N" very poorly; 0·5 *N* H_2SO_4 was a much better solvent for this purpose but unfortunately it tended to dissolve significant amounts of the mercaptide. This led to the washing procedure being examined in more detail, using both human hair and gelatin hydrolysates (the latter containing known amounts of added cystine), in order to test the general applicability of the precipitation and washing procedures adopted.

A human hair hydrolysate (prepared as described in detail later) was concentrated to give a 40 % solution of amino-acids and the cuprous mercaptide was precipitated from two portions (125 and 25 ml.) in the usual way with a slight excess of cuprous oxide. Both precipitates were washed four times with water, in centrifuge pots, the volume used each time being 5 ml. per g. of hair represented. The first wash-water (intense green in colour) contained much nitrogen, but no cystine could be detected. Subsequent washings became paler in colour, the final one (pale blue) containing very little nitrogen and no cystine. Apparently these washings remove only mother liquor trapped in the rather bulky, amorphous mercaptide precipitate.

The mercaptides were then washed with increasing strengths of dilute H_2SO_4 as shown in Table 1.

Table 1. *Solvent action of dilute H_2SO_4 on crude cuprous mercaptide*

Nature of wash liquid	Vol. used ml.	Total N extracted mg.	Cysteine mg.	Cysteine-N mg.	Non-cysteine-N mg.	Cysteine-N as % of total N in extract
		A. Mercaptide from 50 g. of hair				
0·1 *N* H_2SO_4	350	63	2	0·2	62·8	0·4
0·25 *N* H_2SO_4	300	70	15	1·6	68·4	2·4
0·50 *N* H_2SO_4	400	49	140	16·3	32·7	49·8
Water	300	27	75	8·7	18·3	47·5
	Total	209	232	26·8	182·2	14·7
		B. Mercaptide from 10 g. of hair				
0·1 *N* H_2SO_4	50	12	0·5	0·05	12	0·4
0·1 *N* H_2SO_4	50	9	0·5	0·05	9	0·6
0·25 *N* H_2SO_4	50	6	5	0·6	5·4	11·1
0·50 *N* H_2SO_4	50	12	40	4·6	7·4	62·2
	Total	39	46	5·3	33·8	15·7

It is obvious from the above data that the amount of cystine-N lost by washing with 0·5 *N* acid (16·3 mg., i.e. 1·7 % of the accepted cystine-N present, see above) is too large to be neglected. However, 0·25 *N* acid removed only one-tenth this amount of cystine. The data show clearly that acid weaker than 0·25 *N* removes non-cystine-N relatively efficiently without any appreciable loss of cystine. The second experiment (B) confirms the data obtained in the first one and shows that the bulk of the precipitate does not greatly influence the nature of the results.

Exp. 3. Attempted recovery of cystine added to a gelatin hydrolysate

A sample of a cuprous mercaptide from a gelatin hydrolysate (precipitated as described above, using 5 g. Cu_2O for a mixture containing 10 g. hydrolysed gelatin plus 1·0 g. cystine) was washed three times with 75 ml. portions of ice-cold water in a centrifuge pot. As in the case of the mercaptide from the hair hydrolysate, the first wash water was a rich blue-green; the final one was only very pale blue. These washings were added to the main filtrate containing the other amino-acids and the mixture was analysed for total N. No cystine could be detected in this mixture:

	mg.
Total N in original mixture	= 1748
N in filtrate and washings	= 1543
Therefore N in precipitate (by diff.)	= 205
Cystine-N in original mixture	= 116·5
Therefore N precipitated in excess of cystine-N	= 88

The cuprous mercaptide was washed in centrifuge pots three times with 75 ml. portions of very dilute H_2SO_4 (the first two being 0·05 *N*, the third 0·1 *N*), and finally with water. The individual washings were analysed for N, then freed from copper with H_2S and after complete removal of the latter were tested for cystine with cyanide-nitroprusside. Only barely detectable traces of cystine were found although the acid solutions contained much of the non-cystine-N (total of 70 mg.) originally present in the crude mercaptide precipitate. The mercaptide was suspended in water and decomposed with H_2S; the copper sulphide was washed twice with water saturated with H_2S and once with 0·005 *N* H_2SO_4. In this experiment the copper sulphide was not analysed for adsorbed N, although subsequent work has shown that 1·5–2 mg. per g. are usually adsorbed. After exact removal of sulphate from the combined solutions (with $BaCl_2$) the mixture was taken to dryness *in vacuo*. When brought to constant weight over flake NaOH and conc. H_2SO_4, the cysteine hydrochloride weighed 1·272 g. (corrected for aliquots removed for tests) and contained 8·5 % N; weight expected 1·311 g., theoretical N 8·89 %. On dissolving this hydrochloride in water, making it alkaline with ammonia, aerating to a negative —SH test and bringing to the isoelectric point of cystine with acetic acid, 82 % of the cystine originally taken was recovered. However, if the yield be calculated on the weight of crude cysteine hydrochloride oxidized, the recovery was 85·4 %. The difficulties involved in the quantitative recovery of cystine from cysteine by aeration of an ammoniacal solution are well known, having been discussed by Harris [1923] and others. It was decided in future work to investigate other oxidation procedures.

While the weight of the crude cysteine hydrochloride accounted for 97 % of that expected, its N content was low and the cystine recovered from it was only 82 % of that originally taken. The first figure indicated that equation (5) represents the reaction involved, but the latter figure might be considered to be more in agreement with equation (2). To settle the point the following experiment was conducted.

Exp. 4. Recovery of cystine from a mixture of pure amino-acids

The following mixture of pure amino-acids was dissolved in about 150 ml. of *N* HCl and the mixture was twice taken to dryness *in vacuo* to remove the excess mineral acid.

Amino-acid mixture taken

Amino acid	Wt. g.	N content mg.
Glycine	7·009	1307
Alanine	1·013	159·4
Leucine	1·008	107·8
Glutamic acid	0·404	38·4
Arginine	0·402	129·5
Tyrosine	0·204	15·8
Cystine	2·513	292·5
Total	12·553	2050·4

The hydrochlorides were dissolved in water and made up to 50·0 ml. This solution was ground in a mortar with 4 g. red Cu_2O and then 1 g. portions of Cu_2O were added until a slight excess remained; 12 g. in all were required. The mixture was transferred to a large beaker and 15 vol. of ice-water were added gradually with vigorous mechanical stirring. The resulting white granular precipitate settled quickly. There was a marked contrast between this product and the dark-coloured, bulky, amorphous mercaptides obtained from protein hydrolysates. After 30 min. in the refrigerator the precipitate was filtered on a hardened paper. It was washed thoroughly several times by resuspending in 75 ml. of ice-water and sucking dry on a filter. These water washings were added to the main filtrate. This solution contained 1707 mg. N; therefore the N precipitated was 343·4 or 50·9 mg. in excess of the cystine-N.

The cuprous mercaptide was repeatedly washed as above with very dilute H_2SO_4. The details are shown in Table 2. Cystine could not be detected in any of the washings.

Table 2

Extract no.	Vol. of wash liquid ml.	Nature of wash liquid	N content of extract mg.
1	75	0·05 *N* H_2SO_4	12·2
2	75	0·05 *N* H_2SO_4	10·1
3	75	0·067 *N* H_2SO_4	8·5
4	75	0·10 *N* H_2SO_4	10·6
5	75	Water	5·2
			46·6

The cuprous mercaptide was suspended in 250 ml. water and decomposed with H_2S. The mixture was centrifuged and the copper sulphide washed once in a centrifuge pot with 200 ml. H_2S-water, ground in a mortar to a smooth sludge and washed four times more with 0·01 *N* HCl, saturating each time with H_2S. This exhaustive washing is necessary to remove as much cysteine as possible. The dry weight of the copper sulphide was 13·77 g.; it still retained 16·5 mg. N.

The combined cysteine solutions were evaporated to dryness *in vacuo* under nitrogen. The residue was dissolved in O_2-free water and made up to 50 ml. An aliquot of 2 ml. was diluted to 100 ml. and portions were analysed for N and cysteine. The cysteine fraction contained 298 mg. N; by Sullivan's highly specific colorimetric procedure [Sullivan & Hess, 1937] 2·40 g. cysteine were found.

The main cysteine solution was made 1·2 *N* with respect to HCl and oxidized with a strong iodine solution (1·25 *N*) at a temperature below $-5°$ [Lucas & King, 1932]. The iodine was added dropwise until a yellow colour persisted for over 2 min.; 18·3 milli-equiv. were required, corresponding to 2·195 g. cystine in the 48 ml. or 2·29 g. in the whole solution. The oxidation mixture was concentrated *in vacuo* to remove as much HCl and HI as possible. The concen-

trate, which finally changed in colour from yellow to brown (traces of free iodine?) was diluted to 75 ml. and almost neutralized with NaOH. The *p*H was then adjusted to 4·8 with sodium acetate. By performing the neutralization very slowly with vigorous mechanical stirring the cystine was obtained in beautifully formed hexagonal plates. Yield (corrected for aliquots) 2·24 g. (89·3 %); N, 11·68, 11·67 % (calc. 11·65 %). Colorimetric comparison by Shinohara's [1935] method with a repeatedly recrystallized sample of cystine also showed the recovered cystine to be pure.

Since our experience with iodimetric oxidation has shown that the recovery of cystine from cysteine rarely exceeds 95 %, the 89·3 % overall recovery which was found indicates that the cysteine solution obtained from the cuprous mercaptide must have contained practically 95 % of the original cystine (confirmed by analytical data above). This effectively excludes reactions of the type shown in equations (1) and (2) and establishes the validity of the assumptions involved in equation (5).

Removal of cystine from keratin hydrolysates

Human hair from a barber's shop, carefully picked over to remove gross impurities, was washed with six changes of distilled water to eliminate dust and other extraneous matter. After being air-dried it was washed in five changes of pure light petroleum, refluxed with several changes of alcohol and finally extracted for some hours with ether in a large Soxhlet apparatus. It was dried in a current of warm dry air (35–40°) and kept in tightly stoppered bottles.

The hair thus prepared had a moisture content of 8·06 % (constant weight over P_2O_5 *in vacuo* at room temperature for 1 month), ash 0·50 %, N 16·2 %, S 5·00 % (the two latter calculated on an ash- and moisture-free basis).

Preliminary experiments showed that boiling with 8–9*N* HCl on a sand bath caused rapid liberation of amino groups during the first hour, a distinct slowing of the process by the third hour, a gradual increase to a maximum figure at 10·5–12 hr. and then a very gradual decrease over a very long period. The rate of liberation of cystine was also followed, using Lugg's [1933] modification of Sullivan's procedure, and a maximum was noted at about 6–7 hr., in good agreement with the findings of Merrill [1921] and Rimington [1929] who made similar studies on wool. The maximum amino-N by Van Slyke's [1911; 1913] method was 72–79 % of the total N in different hydrolysates, although Kendrick & Hanke's [1940] potassium iodide modification gave figures about 6 % lower. This latter method is claimed to give essentially correct figures for glycine, glycyl peptides and cystine, all of which give high results by the unmodified procedure. The cystine figure found at the end of the hydrolysis was 16·4 %, in good agreement with Vickery & Block's [1931] figure of 16·5 % for human hair.

CO_2 and H_2S are given off during the hydrolysis, about 350 mg. of the former and 7 mg. of the latter per 100 g. hair, after 11 hr. boiling.

N distribution was determined on one hydrolysate with the results shown in Table 3.

The isolation of cystine was carried out on a hydrolysate from 328 g. hair (equivalent to 300 g. ash- and moisture-free keratin) refluxed with 1600 ml. 9*N* HCl on a sand bath for 11 hr. After one evaporation *in vacuo* to remove excess HCl the hydrolysate was diluted, melanin was filtered off, washed five times with dilute HCl and the combined filtrate and washings were evaporated to a thick syrup three times before making up to 1200 ml. An aliquot of 1 ml. was removed, diluted to 50 ml. and used for N and cystine determinations.

Table 3. *Nitrogen distribution in hair hydrolysate*

(Total N in hair keratin = 16·04 %)

	% of total N	
	Observed	Corrected
Melanin-N	0·56	0·56
Amide-N	7·22	7·22
Basic N	33·4	35·8*
Dicarboxylic N	11·5	11·5
Monoamino-acid-N + non-amino-N	47·32	44·9

* Corrected for solubility of phosphotungstates (Plimmer).

The remaining solution was ground in a mortar with 300 g. red cuprous oxide. Some of the oxide remained unchanged even after 30 min. grinding. The mixture was added slowly with constant stirring to 12 l. of ice-water; after being left in the refrigerator for 2 hr. the greyish white mercaptide was filtered off by suction. It was thoroughly washed by suspending it three times in 500 ml. portions of ice-water, rubbing to a smooth suspension and filtering with suction to a dry hard cake. The total filtrate contained 37·450 g. N, from which figure the N content of the crude mercaptide can be calculated to be 10·69 g., a value considerably in excess of the 5·73 g. N expected if one calculates from the cystine figure obtained by the Sullivan method. The direct cuprous oxide precipitation as used does not possess very great specificity.

The crude mercaptide precipitate was washed as above with three 500 ml. portions of 0·10 *N* H_2SO_4 and given a final wash with 700 ml. cold water. The N thus removed was 1·24 g. The mercaptide was suspended in 3 l. of water and copper removed with H_2S. The copper sulphide was washed four times by suspending in 3 l. of water each time, saturating with H_2S and centrifuging. The dried sulphide weighed 380 g. and contained 697 mg. N.

The filtrate (cysteine hydrochloride solution) was concentrated *in vacuo* and several hundred milli-equiv. of HCl were thus removed. The solution was made up to 500 ml. and 1 ml. was taken for N determinations. The solution, which was found to contain about 3·5 g. non-cystine-N, was ground in a mortar with a slight excess of Cu_2O (100 g.) and the suspension was poured slowly with constant stirring into 15 vol. of ice-water. After 2 hr. in the refrigerator, the white granular mercaptide was filtered with suction, washed by suspending in 1 l. of ice-water and again sucked to a dry hard cake. The filtrate contained 2·71 g. non-cystine-N and traces (125 mg.) of cystine. Other small losses of cystine were noted: 250 mg. in the original filtrate of the other amino-acids and 37 mg. in the acid washings of the mercaptide. The total loss thus accounted for was 412 mg. or less than 1 % of the cystine started with.

The reprecipitated mercaptide was decomposed with H_2S and the chocolate-brown Cu_2S was washed as described above. The dried sulphide weighed 128 g. and contained 189 mg. N. The filtrates were concentrated *in vacuo* under N_2 and made to 500 ml. Small aliquots were removed for analysis. The main solution was boiled with 3 g. charcoal to remove a slight brown coloration and the filtrate was concentrated *in vacuo* under N_2 to incipient crystallization. It was warmed slightly, transferred to a beaker and chilled, upon which it formed an almost solid mass of crystals. These were removed on a fritted glass filter, washed with cold conc. HCl and dried *in vacuo* over conc. H_2SO_4 and flake NaOH. A second crop was obtained in the same manner from the filtrate, after concentration. The filtrate was brought to dryness in the same way; the residue was called crop III.

Crystalline cysteine hydrochloride isolated from 300 g. hair

Crop	Wt. g.	N* %	S* %	Yield (calc. as cystine)
I	30·6	8·86	20·14	7·86
II	13·2	8·93	19·45	3·40
III	22·4	9·83	16·38	4·64 (calc. from S)
Total	66·2			15·90

* Calc. for cystéine hydrochloride: N, 8·89; S, 20·3%.

The analytical data on crops I and II were considered satisfactory and the material was accepted as essentially pure cysteine hydrochloride. Crop III, however, was grossly impure and calculations based on the S and N contents indicated contamination with material very high in N. A strongly positive Sakaguchi test was obtained.

By recrystallizing crop III from concentrated HCl another 8·3 g. of fairly pure cysteine hydrochloride were obtained. The separation of arginine from the remaining cysteine was effected as follows. The filtrate was evaporated to dryness to remove excess HCl, taken up in water and treated with hot saturated Ag_2SO_4 solution until an excess of silver ion was present. The mixture was brought to *p*H 5·5–6 with baryta; the precipitate of silver cysteine, silver chloride and barium sulphate was removed and washed with water. From the filtrate, which gave no test for cystine, 0·97 g. arginine was isolated as flavianate. Although the filtrate still contained about 100 mg. N, no other amino-acid was identified.

The silver precipitate was decomposed by boiling several times with an excess of HCl and the resulting cysteine hydrochloride solution was taken to dryness *in vacuo*. It was dissolved in water, made slightly alkaline with ammonia, treated with a drop of dilute $FeCl_3$-$CuSO_4$ mixture and aerated. After recrystallizing the 6 g. crude amino-acid (N, 11·1; S, 21·4%) several times, only about 4·2 g. of pure cystine (N, 11·64%) were obtained. About 5 g. of crop III were thus left unaccounted for by the products isolated and on the basis of the S content of crop III about 3 g. of the uncrystallizable material were cysteine or some product derived from it.

Discussion

The recovery from the hair hydrolysate of pure, crystalline cystine (14·8%) as such, or as cysteine hydrochloride, accomplished in this experiment exceeds considerably any isolation figure previously reported in the literature. As far as the authors are aware, the highest yield of analytically pure cystine hitherto isolated from a human hair hydrolysate is 10·7% [Vickery & Leavenworth, 1929]. Buchtala [1907] claimed to have isolated 14·5% but this figure was in part arrived at by calculation from the S content of very impure fractions; the same remarks apply to Barritt's figure of 13·0%. Barritt [1927] and others [Hopkins, 1929; Harris, 1923; Rimington, 1929; Toennies & Bennett, 1935–6] have commented on the usual inability to isolate more than 60–70% of the cystine-sulphur of keratins or any other protein as reasonably pure cystine. The cuprous oxide procedure described above affords a considerable improvement in this regard. The cystine figure of 14·8% (material obtained in pure crystalline form) corresponds to 79% of the keratin-S; if the S in impure fractions of cystine be included the fraction accounted for reaches 85%. Further improvements in

the method will doubtless enable still larger proportions of cystine to be isolated in pure crystalline form.

The use of cuprous oxide not only makes it possible to recover the cystine in excellent yield and high purity but separates the cystine quantitatively from the other amino-acids and in such a way that the remainder of the hydrolysate is in a form suitable for the application of other isolation procedures. The value of this reagent is further enhanced by the fact that it removes from the dark hydrolysate practically all of the chloride ion and also colloidal colouring matter which tends to inhibit crystallization [cf. Abderhalden & Fuchs, 1908]. After removal of copper from the main filtrate containing the other amino-acids, an almost water-clear or pale straw-coloured solution is obtained.

In view of the results obtained in this study a note of warning concerning the analytical use of cuprous oxide as a reagent for cysteine seems warranted. Graff *et al.* [1937] claim that the specificity of cuprous oxide for cysteine is so marked that when it is added to a reduced protein hydrolysate the precipitate obtained is pure cysteine cuprous mercaptide. The analytical data presented in their paper certainly appear to support that statement, but none of our experiments have given quite such desirable results. While the direct use of cuprous oxide on an unreduced solution cannot be compared with their procedure, the reprecipitation of the cysteine fraction should be more nearly comparable, yet even here 8·8% of the N in the mercaptide was found to be non-cysteine-N. Experiments with pure amino-acids (as hydrochlorides) either alone or mixed with cysteine, proved that considerable quantities of some, particularly glycine and arginine, are precipitated by cuprous oxide under the conditions used by us. While these findings do not necessarily disprove the results of Graff *et al.* [1937], since our materials and conditions are not identical with theirs, considerable suspicion is cast upon the reliability of cuprous oxide as a highly specific precipitant for cysteine in protein hydrolysates. Certainly little trust should be placed in the accuracy of cysteine determinations based upon the nitrogen content of mercaptide precipitates.

However, in spite of its lack of complete specificity, cuprous oxide should be considered a valuable reagent because of its relative specificity for sulphydryl compounds. Its specificity has proved sufficient to be of great value in improving the isolation of cysteine, just as it did in the case of glutathione [Hopkins, 1929; Pirie, 1931]. Its value as an analytical reagent depends upon its use in the manner described by Vickery & White [1933], viz. determination of the organic sulphur in the cuprous mercaptide precipitate.

Summary

1. Cuprous oxide can be used to remove cystine quantitatively from protein hydrolysates.

2. Since the cuprous oxide procedure to remove cystine does not require the use of alkalis or inorganic salts, the remainder of the hydrolysate is in a suitable condition for the application of other isolation procedures.

3. Cuprous oxide simultaneously removes most of the chloride ion and all of the colloidal colouring matter from the hydrolysate.

4. Pure crystalline cystine (or cysteine hydrochloride) may be readily obtained in excellent yield from the cuprous mercaptide precipitated.

5. The yield of pure cystine isolated from a human hair hydrolysate has been considerably increased above the usual amount obtained by fractional crystallization of neutralized hydrolysates.

This work was aided in part by a grant from the Banting Research Foundation to the Department of Medical Research. Grateful acknowledgement is made to Miss Margaret Wheatley and Mr William J. Watson for many careful analyses and technical assistance.

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162. THE EFFECT OF X-RAYS ON ENZYMES

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(*Received 6 August 1940*)

Because of the importance of enzymes for cell metabolism many have investigated the action of radiation on crude enzymes but only a few have used purified ones. The general findings arrived at in such investigations can be briefly summarized by repeating the conclusions of a recent review by Scott [1937] of the biological effects of X- and γ-rays: "The evidence shows that X- and γ-rays only influence enzymes when the dose is enormous. Cell division is inhibited by doses which have little or no destructive action on enzymes and therefore the inhibition is produced by a process in which enzymes play no important part."

It has been stated further that X- and γ-rays in doses of the order of 100,000 r. have an indiscriminate destructive effect on all living tissues causing a massive physico-chemical change in the protoplasm. Small doses however are supposed to act in a qualitatively different manner.

This paper deals with the action of X-rays on two enzymes:

(i) The crystalline enzyme carboxypeptidase which was isolated for the first time by Anson [1936–7, 1]. This enzyme is a water-insoluble protein which attacks chloroacetyltyrosine and peptic digests of proteins in the presence of formaldehyde.

(ii) A partly purified sample of polyphenoloxidase.

Experimental

Carboxypeptidase

The enzyme was prepared by the method of Anson with slight modifications. Anson recommends two ways of preparation either from the fluid which exudes when fresh frozen pancreas is allowed to thaw slowly, or by extraction of ordinary commercial pancreas. Since the facilities for the first were not available and the ordinary commercial frozen pancreas obtainable locally proved unsuitable and too expensive, a combination of the two methods was adopted.

Batches of 12 lb. of bovine pancreas were collected weekly, frozen hard immediately after the animal's death, sliced and kept in cold storage at $-9°$. It was then spread on trays, allowed to thaw slowly in a refrigerator at 5° for 20 hr. and minced. The subsequent treatment followed Anson's instructions as given for ordinary commercial pancreas. The yield of the preparation varies considerably for no apparent reason. It was found several times that the precipitation after dialysis remained incomplete, leaving a large part of the active material in a colloidal state. This difficulty was overcome by adding saturated $Ba(OH)_2$ until the fluid became just orange to phenol red. At this reaction a precipitate of the active material formed.

The experiments to be described were performed with a uniform batch of white crystals which were obtained after twice recrystallizing an originally good crop of crystals. The crystals were suspended in redistilled water with toluene as preservative and kept as stock suspension in a refrigerator, the activity remaining

unchanged over 12 months. The activity was the same as given by Anson, i.e. the amount of the crystalline enzyme containing 1 mg. N corresponded to 0·103 $[C.P.u.]^{P.D.E.}$ where $[C.P.u.]^{P.D.E.}$ denotes carboxypeptidase units when a peptic digest of edestin was used. With one exception the substrate used throughout the experiment was a peptic digest of edestin (Hopkin and Williams, Ltd.) prepared according to Anson. The digestion took place at 25°, if not stated otherwise.

Titration of enzyme activity. The titrations were carried out with 0·02 *N* NaOH, the burette being protected against CO_2 by soda lime tubes. The procedure as given by Anson [1936–7, 2] was closely adhered to. The pipetting of the substrate solution has been done with the greatest accuracy.

General procedure. The suspension of crystals was thoroughly shaken in order to distribute the fine crystals evenly. The required amount was taken out immediately before the crystals had time to settle, diluted with glass-distilled water to the desired strength and *N*/10 NaOH added drop by drop, pausing for about 1 min. after every two drops and constantly shaking the flask until all crystals were dissolved and the reaction was pink to phenolphthalein. After about 5 min. a water-clear solution of the crystals was obtained. Half of the solution was kept as control, the other half exposed to the X-radiation in flat bottles. When the desired dose of radiation was given a sufficient volume was taken out for 4 titrations of the remaining activity and 4 blank titrations for each point of the curve. In the experiments with highly diluted solutions the number of titrations had to be reduced to 3 or even 2 because the volume of fluid which could be exposed to radiation was limited by the diameter of the X-ray tube window. For the irradiations where high doses or high dosage rates were required, radiation of half value layer 6 mm. Cu from a 450 kV. Metropolitan-Vickers continuously evacuated X-ray tube with a cooled window was used. For the other irradiations X-rays of half value layer 1·7 mm. Cu from a 250 kV. Metropolitan-Vickers tube, as described by Allibone & Bancroft [1934], were used. The dosage rate from the first tube was 1000 r. per hr. whilst from the second it was varied for the different experiments. The total dose was measured in every case by a calibrated Hammer Dosimeter.

The same stock suspension of crystals was used for further dilutions throughout. The estimation of the activity for the various dilutions was carried out under the conditions laid down by Anson. The digestion time of the substrate and the amount of enzyme solution used was adapted to the concentration of the enzyme solution of each particular experiment (see Table 1, columns 5 and 6). The lower the concentration of the enzyme solution the more the digestion time and the volume of the enzyme solution in the digestion titration had to be increased. The volume of the carboxypeptidase solution, however, never exceeded 2·5 ml. in order to keep the total volume of the digestion titration within the prescribed limit. When the volume of the carboxypeptidase solution for titration was under 2·5 ml. it was made up to 2·5 ml. by adding glass-distilled water.

Effects of X-radiation on carboxypeptidase solutions of different concentrations

The results of these experiments are shown in Figs. 1–4 and in Table 1 giving the experimental details and the calculations.

In calculating the carboxypeptidase units of the solution the values corresponding to the number of ml. of 0·02 *N* NaOH used in the titration (Table 1, columns 3 and 4) are read from the standard curve of Anson's paper, and then divided by the number of ml. of CP solution used (Table 1, column 6), and by the digestion time (Table 1, column 5).

The absolute number of the carboxypeptidase units inactivated by irradiation is found by converting the ml. of NaOH corresponding to the original activity (column 3) and to the activity left after irradiation (column 4) into carboxypeptidase units (columns 7 and 8), and then taking the difference between these two values (column 9).

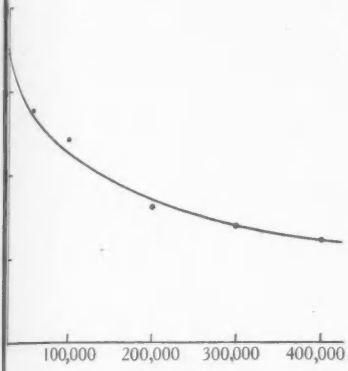

Fig. 1.

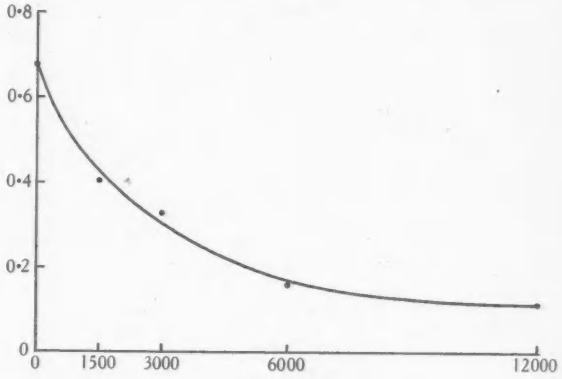

Fig. 2.

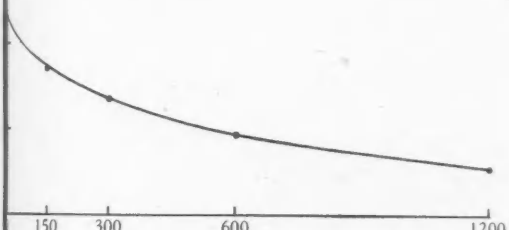

Fig. 3.

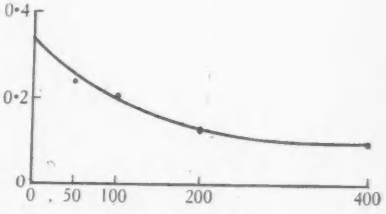

Fig. 4.

Fig. 1. Exp. A of Table 1.
Fig. 2. Exp. B of Table 1.
Fig. 3. Exp. C of Table 1.
Fig. 4. Exp. D of Table 1.

Abscissae: Dose in roentgen. Ordinates: Activity in ml. $N/50$ NaOH.

The reaction of the enzyme with its substrate is not strictly linear, i.e. decreasing the amount of enzyme or the digestion time leads to a comparatively smaller decrease in the activity of the enzyme. Therefore all four curves show a diminishing slope and the dilution of the enzyme by volume is not quite proportional to the dilution as indicated by the values of the enzymic activity (Table 1, columns 1 and 2). Because of this it is necessary when comparing the four curves with each other to choose a point on each curve representing the same percentage of destruction of the original activity. In Table 1, the points chosen for the curves A to D are where 25 or 50 % of the original activity is lost.

Table 1. *Inactivation of carboxypeptidase solutions of different concentration*

	1	2	3	4	5	6
Exp.	Dilution by volume	Dilution by units	Activity unirradiated ml. 0·02 *N* NaOH	Activity left after inactivation ml. 0·02 *N* NaOH	Digestion time min.	C.P. solution used per titration ml.
			For 25 % inactivation			
A	×1	×1	0·69	0·52	1×10	0·2
B	×62	×62·5	0·68	0·51	6×10	2·0
C	×172	×210	0·46	0·35	12×10	2·5
D	×345	×884	0·34	0·26	30×10	2·5

	7	8	9	10	11
Exp.	Column 3 in $10^6 \times$ C.P.u./ml./10 min.	Column 4 in $10^6 \times$ C.P.u./ml./10 min.	Difference between columns 7 and 8	X-ray dose in r. inactivating units of column 9	$10^6 \times$ C.P.u./ml. inactivated per 1000 r./10 min.
A	8000	5400	2600	60,000	43
B	128	88	40	800	50
C	31	23	8	145	55
D	9·1	6·8	2·3	45	50

	1	2	3	4	5	6
Exp.	Dilution by volume	Dilution by units	Activity unirradiated ml. 0·02 *N* NaOH	Activity left after inactivation ml. 0·02 *N* NaOH	Digestion time min.	C.P. solution used per titration ml.
			For 50 % inactivation			
A	×1	×1	0·69	0·35	1×10	0·2
B	×62	×62·5	0·68	0·34	6×10	2·0
C	×172	×210	0·46	0·23	12×10	2·5
D	×345	×884	0·34	0·17	30×10	2·5

	7	8	9	10	11
Exp.	Column 3 in $10^6 \times$ C.P.u./ml./10 min.	Column 4 in $10^6 \times$ C.P.u./ml./10 min.	Difference between columns 7 and 8	X-ray dose in r. inactivating units of column 9	$10^6 \times$ C.P.u./ml. inactivated per 1000 r./10 min.
A	8000	3450	4550	185,000	25
B	128	57	71	2,500	28
C	31	15·7	15·6	420	37
D	9·1	4·7	4·4	125	35

Column 9 gives the absolute amounts of enzyme in carboxypeptidase units/ml. which have been inactivated by irradiation. Column 10 gives the corresponding dose of inactivating X-rays and column 11 the absolute amount of enzyme in carboxypeptidase units/ml. inactivated per 1000 r.

This column shows that the number of carboxypeptidase units/ml. inactivated by a given number of roentgens and for a given percentage of inactivation is a constant figure for a wide range of X-ray doses, and from columns 2 and 10 it is evident that the same percentage inactivation is obtained by a wide range of X-ray doses depending on the concentration of the carboxypeptidase solution. This applies to 25 % and also to 50 % inactivation as the constant in column 11 shows. That this constant is lower for 50 % than that obtained for 25 % inactivation (Table 1) follows from the non-linearity of the enzyme reaction.

Effect of X-radiation on carboxypeptidase solution acting on its substrate during radiation

The concentration of the enzyme solution was C.P.u./ml. $= 10^{-6} \times 59{\cdot}4$ in this experiment (2·6 ml. enzyme for titration, digestion time $= 12 \times 10$ min.): irradiation of this solution by 10,000 r. reduces the activity to $10^{-6} \times 7{\cdot}7$ u./ml., whereas the enzyme acting on its substrate during irradiation had an activity of $10^{-6} \times 67{\cdot}4$ u./ml. which is even slightly higher than the activity of the original enzyme solution. The temperature during irradiation was 20°.

The substrate irradiated alone in control experiments did not show any change.

The carboxypeptidase solution of $10^{-6} \times 59{\cdot}4$ u./ml. would be inactivated to about 77 % by a dose of about 1200 r. which was delivered in the first 3 min., during which time the enzyme could not have reacted to any extent since the digestion time for this concentration was 2 hr. To be sure of the ineffectiveness of the radiation on the enzyme whilst mixed with its substrate, radiation was continued up to a total of 10,000 r. This experiment showed clearly that no inactivation takes place whilst the enzyme is acting on its substrate during irradiation.

The same type of experiment with chloroacetyltyrosine as substrate had an analogous result. The figures are:

Activity of the original enzyme solution $[\text{C.P.u.}]^{CT}_{ml.}$	$= 10^{-6} \times 16{\cdot}7$
Activity after 10,000 r.	$= 10^{-6} \times 4{\cdot}67$
Activity after 10,000 r. irradiated whilst mixed with substrate	$= 10^{-6} \times 16{\cdot}7$

Effect of X-rays on polyphenoloxidase

It was of interest to test whether the results obtained on carboxypeptidase would also hold for an oxidizing enzyme containing a heavy metal. The sample of purified polyphenoloxidase, made from mushrooms by the method of Keilin & Mann [1938], was kindly given to me by Dr H. Jackson of Manchester University. The course of reaction of polyphenoloxidase with its substrate pyrocatechol falls off very rapidly after a short linear start. This can be improved to a certain extent by adding aniline which removes the reaction product of the enzymic activity by forming insoluble dianilo-*o*-quinone. In spite of this the necessity of extending the reaction time between enzyme and substrate with increasing dilution of the enzyme solution soon leads to such a diminution of enzymic activity that the limit of the method for estimating the activity is reached. It is, however, possible to demonstrate that polyphenoloxidase behaves in an analogous way within the range of 20,000–2500 r.

The estimation of the activity was carried out according to Keilin & Mann [1938] in Barcroft-Warburg manometers with conical vessels equipped with side bulb and inner compartment. The various dilutions used in the experiments were made up with distilled water from a stock solution of polyphenoloxidase of a Q_{O_2} value of 8461, and a dry weight of 1·04 mg./ml. The conditions of the experiment (Table 2) were as follows:

The main chamber of the manometers received 2·6 ml. of 0·03 *M* phosphate buffer solution (*p*H 7·3), of which 30 ml. contained 100 mg. pyrocatechol and two drops of aniline. The side bulb contained 0·5 ml. enzyme solution, the inner compartment 0·2 ml. of a 20 % KOH solution soaked into a roll of filter paper. The temperature of the water bath was 20°.

Table 2. *Effect of X-rays on various concentrations of polyphenoloxidase*

Time min.	Unirradiated	X-ray dose 10,000 r.	X-ray dose 20,000 r.	ml. enzyme: ml. H_2O
5	5·5	5·3	2·8	0·1:20
10	11·5	8·6	6·1	
20	15·7	13·9	6·9	
	Unirradiated	5000 r.	10,000 r.	
10	8·7	2·4	0	0·1:40
20	11·3	4·2	0	
40	12·5	5·4	0	
	Unirradiated	2500 r.	5000 r.	
20	5·6	2·1	0	0·1:80
40	7·0	3·8	0	

It can be seen from Table 2 that for a dilution of the enzyme solution of 1:200, 10,000 r. reduce the original activity slightly, and 20,000 r. to about one-half. For a dilution of 1:400, 5000 r. inactivate about 65% and 10,000 r. inactivate it completely. This shift of the inactivating X-ray dose to lower levels continues on doubling the dilution again. In other words, the dependence of the percentage inactivation on the dilution for a given dose of X-rays is principally the same as the effect of radiation on carboxypeptidase though it is not possible to work out a constant for the reasons mentioned above. Experiments in which the dilution was once more doubled showed that no O_2 consumption took place even in the unirradiated control, making it impossible to perform experiments with doses of X-rays still lower than 2500 r.

Discussion

The reason why earlier investigators have found either no effect of radiation on enzymes or only after the application of enormous doses is that they did not use sufficiently low concentrations of enzymes and also probably that they used too impure enzymes.

The fact that a constant amount of enzyme is inactivated by a given dose of radiation independent of the concentration recalls results by Fricke & Hart [1935], when they obtained oxidation of ferrocyanide to ferricyanide by irradiating their aqueous solution. They found the number of equivalents oxidized per litre and per 1000 r. to be constant and independent of the concentration, and concluded that these transformations are due to "X-ray-activated" water molecules. This is only another way of expressing the same facts and we refrain from assuming any hypothetical mechanism of how the X-rays act on enzymes.

The fact, however, that the two different types of enzymes examined are affected similarly may justify the assumption that many more may respond in a similar way.

In view of the results obtained with dosages ranging from 400,000 r. to values well below those normally used in radiotherapy there is now no reason to assume that only very big doses have an effect on enzymes or that big doses act qualitatively differently from very small ones, nor is it justified to exclude enzymes from playing an important part in explaining the radiation effects on living tissues. On the contrary the concentration of the enzyme and its state during irradiation will be a factor in the action of X-rays on living tissue, the importance of which will vary with the degree to which a particular enzyme is a limiting factor in cell metabolism. Those cells, or even certain phases in their life (e.g.

mitoses) for which the action of certain enzymes is the limiting factor would then be spoken of as showing greater radiosensitivity than other cells. It does not seem to be without significance in this connexion that the increase of the percentage inactivation of enzymes with increasing dilution coincides with the well-established fact of a higher water content and greater radiosensitivity of embryonic tissues compared with the lower water content and lower radiosensitivity of adult tissues. Finally the ineffectiveness of X-rays on carboxypeptidase irradiated in the presence of its substrate may, if it can be generalized, furnish an explanation of the so-called delayed action of X-rays in the sense that the reserves of unused enzyme in the cell are inactivated by radiation, whilst that part of the enzyme already in action remains unaffected by radiation.

Summary

1. The effects of X-rays on crystalline carboxypeptidase and on partly purified polyphenoloxidase have been examined.

2. The percentage inactivation of each of these enzymes is a function of the concentration of the enzyme for a given dose of radiation.

It can be shown for carboxypeptidase that a definite amount of radiation energy absorbed corresponds to a constant amount of enzyme inactivated. In consequence the inactivating dose of X-rays decreases with decreasing concentration of the enzyme for a given percentage inactivation to levels far below those normally used in radiotherapy.

3. X-rays have a qualitatively uniform effect on carboxypeptidase over the whole range from very small doses to doses 1000 times as large.

4. Inactivation of carboxypeptidase does not take place when the enzyme is acting on its substrate during irradiation, whereas the enzyme irradiated without its substrate is inactivated to 85 %.

5. The biological significance of the experimental results is discussed.

The author wishes to thank Mr W. K. Johnstone and Mr C. E. O'Neill of the Manchester Municipal Cold Stores for their co-operation in supplying suitable pancreatic glands.

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163. TRIOSEPHOSPHATE DEHYDROGENASE OF *BACTERIUM COLI*

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(*Received 19 August 1940*)

THE Booth-Green wet-crushing mill for bacteria has been used previously to prepare a cell-free juice from *Bacterium coli* in which the components of enzyme systems may be studied. In the present paper a juice has been prepared containing the system which oxidizes 3-phosphoglyceraldehyde.

For yeast it has recently been shown that the actual substrate is 1:3-diphosphoglyceraldehyde [Warburg & Christian, 1939]. The evidence in this paper does not conflict with the view that the same is true of *Bact. coli*. The object of this investigation was the partial purification of the triosephosphate dehydrogenase of *Bact. coli* and its comparison with the oxidizing enzyme of yeast fermentation. It has already been shown that the reducing enzyme, alcohol dehydrogenase, occurs in *Bact. coli* [Still, 1940].

I. *Preparation*

The organism used was *Bact. coli commune* Escherich (National Collection of Type Cultures No. 86). The organism was grown on agar with tryptic digest of casein and mineral salts. After washing the cells were crushed in the mill [Booth & Green, 1938], centrifuged for 30 min. at 3000 r.p.m., the supernatant heated to 55° and centrifuged for 40 min. at 12,000 r.p.m. This supernatant was dialysed against running water. This procedure is essentially that previously followed [Still, 1940]. It was found that in the case of the triosephosphate dehydrogenase more prolonged dialysis was required to remove coenzyme I, and that it was an advantage to dilute the crushed cells more before centrifuging.

The activity of the enzyme was measured by Thunberg tube technique and manometrically by Warburg manometers. The coenzyme I used was made by a modification of the method of Green & Brosteaux [1936]. The hexosediphosphate was estimated with zymohexase from rabbit skeletal muscle [cf. Herbert *et al.* 1940].

II. *Zymohexase*

The enzyme solution prepared as above converted hexosediphosphate into phosphoglyceraldehyde. This was shown as follows: 2·0 ml. enzyme, 1·0 ml. *M*/2 NaCN, 1·0 ml. water and 2·0 ml. *M*/20 hexosediphosphate were added to each of two tubes. To one tube 0·32 ml. 40% trichloroacetic acid was added immediately. The other tube was incubated at 38° and then 0·32 ml. 40% trichloroacetic acid was added. Two controls without hexosediphosphate were also set up and treated similarly. After standing at 0°, the contents of the tubes were filtered through Whatman No. 40 paper. Changes in the amount of inorganic and alkali-labile phosphate were determined by the method of Lohmann & Jendrassik [1926]. Phosphate rendered labile by treatment with *N* NaOH for 20 min. at room temperature was considered to be derived from phosphoglyceraldehyde.

The results summarized in Table 1 indicate the presence of zymohexase and phosphatases in the enzyme solution. In view of the instability of phosphoglyceraldehyde hexosediphosphate was used as the substrate in all the subsequent experiments.

Table 1. *Demonstration of zymohexase in enzyme solution*

		Inorganic P (mg.)	Alkali-labile P (mg.)
Enzyme + cyanide + hexosediphosphate:	Initial	0·6	0
	Final	1·05	3·88
Enzyme + cyanide + hexosediphosphate:	Initial	0·19	0
	Final	0·31	0·08

III. *Components of the oxidation system*

The enzyme solution required the addition of coenzyme I and either phosphate or arsenate in order to reduce methylene blue in the presence of hexosediphosphate (Table 2). By analogy with the yeast enzyme this was taken to mean that 3-phosphoglyceraldehyde is first phosphorylated or arsenylated to 1:3-diphosphoglyceraldehyde or 1-arseno-3-phosphoglyceraldehyde both of which are substrates for the enzyme [Warburg & Christian, 1939]. The relative instability of the resulting arsenophosphoglyceric acid probably accounted for the greater activity in the presence of arsenate.

Table 2. *Demonstration of anaerobic oxidation of phosphoglyceraldehyde*

	Additions to tubes (ml.)					
	1	2	3	4	5	6
Enzyme	1·0	1·0	1·0	1·0	1·0	1·0
Phosphate *M*/15 *p*H 7·4	—	1·0	—	1·0	1·0	1·0
Arsenate *M*/10 *p*H 7·4	—	—	0·7	—	—	0·7
Adenylic acid 1·0 mg./ml.	—	—	—	0·5	—	—
Coenzyme I 5 mg./ml. 6·8% pure	0·1	0·1	0·1	0·1	0·1	—
Methylene blue 0·5%	0·2	0·2	0·2	0·2	0·2	0·2
Hexosediphosphate *M*/20	0·2	0·2	0·2	0·2	—	0·2
Water	1·6	0·6	0·9	—	0·8	—
Reduction time in min.	>120	17	2¼	18	>120	>120

The enzyme was not destroyed when precipitated with acetone in the cold. An acetone preparation relatively free from coenzyme dehydrogenase was made as previously described [Still, 1940] and its activity tested with and without added coenzyme dehydrogenase (diaphorase), the latter being provided in the form of milk flavoprotein (which rapidly catalyses the oxidation of reduced coenzyme I by methylene blue). The results in Table 3 indicate that coenzyme dehydrogenase is a component of the system in which methylene blue is reduced by phosphoglyceraldehyde.

Table 3. *Effect of flavoprotein on acetone preparation*

	Q_{MB}
Enzyme solution	75
Acetone powder	7·2
Acetone powder + milk flavoprotein	36·2

Q_{MB} = μl. H_2 transferred to methylene blue per hr. per mg. dry weight of enzyme.

IV. *Reaction with molecular oxygen*

In the presence of methylene blue the system reacts with molecular O_2. Table 4 shows that for this reaction also coenzyme I and either phosphate or arsenate are necessary. Fig. 1 shows the extent of the reaction in the presence of phosphate and arsenate.

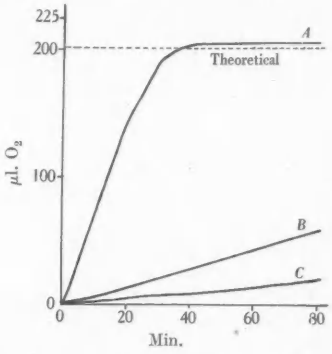

Fig. 1. Effect of arsenate (*A*) and phosphate (*B*) on the oxidation of phosphoglyceraldehyde. Warburg manometers containing 1·0 ml. enzyme, 0·2 ml. *M*/22 hexosediphosphate, 0·5 ml. 0·5% methylene blue, 1 mg. coenzyme I (14% pure) and 0·1 ml. *M* NaF. Total volume 3·2 ml. *A* contained 0·3 ml. *M*/5 arsenate, *B* contained 1·0 ml. *M*/15 phosphate, and *C* no additions.

Table 4. *Aerobic oxidation of phosphoglyceraldehyde. Additions to Warburg manometers (ml.)*

Enzyme	1·0	1·0	1·0	1·0	1·0	1·0
Phosphate *M*/5 *p*H 7·7	—	0·5	—	—	—	—
Arsenate *M*/5 *p*H 7·7	—	—	0·5	0·5	0·5	0·5
Coenzyme I 5 mg./ml. 27% pure	0·2	0·2	0·2	—	0·2	0·2
Methylene blue 0·5%	0·5	0·5	0·5	0·5	0·5	—
Hexosediphosphate *M*/20	0·4	0·4	0·4	0·4	—	0·4
Water	1·0	0·5	0·5	0·7	0·9	1·0
Sodium fluoride *M*	0·1	0·1	0·1	0·1	0·1	0·1
μl. O_2 taken up in 10 min.	4·0	26	154	0	7	23
20 ,,	8·5	44·5	284	0	10	44
40 ,,	18·0	77·5	416	5	19	85

The enzyme preparation also oxidized glyceraldehyde under the same conditions, but only to a small extent (Table 5). This may mean that either the glyceraldehyde dehydrogenase or the necessary phosphorylating enzymes have been inactivated by the treatment in preparing the extract. From the work of

Table 5. *Aerobic oxidation of glyceraldehyde. Additions to Warburg manometers (ml.)*

Enzyme	1·0	1·0	1·0	1·0	1·0	1·0
Phosphate *M*/5 *p*H 7·7	—	0·5	—	—	—	—
Arsenate *M*/2 *p*H 7·7	—	—	0·2	0·2	0·2	0·2
Coenzyme I 5 mg./ml. 27% pure	0·2	0·2	0·2	—	0·2	0·2
Methylene blue 0·5%	0·5	0·5	0·5	0·5	—	0·5
Glyceraldehyde 1·5%	0·2	0·2	0·2	0·2	0·2	—
Sodium fluoride *M*	0·1	0·1	0·1	0·1	0·1	0·1
Water	1·0	0·7	1·0	1·2	1·5	1·2
Oxygen uptake μl. in 20 min.	15	22·5	32	16	10·5	8·6

Warburg and Christian, however, it would seem that glyceraldehyde is slowly oxidized by the phosphoglyceraldehyde enzyme, and these authors do not attach much physiological significance to the oxidation of glyceraldehyde.

The aerobic oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid by the enzyme system in the presence of arsenate is complete (Fig. 1). NaF was added to inhibit the conversion of phosphoglyceric acid into phosphopyruvic acid.

V. *Mechanism of reaction*

The spectrophotometric method was used to show the oxidation and reduction of coenzyme I in the system Green & Dewan [1937]. The reduction of coenzyme I could be demonstrated only in the presence of arsenate (Table 6).

Reduction of coenzyme I. Five Thunberg tubes were set up as shown in Table 6. The contents were incubated at 38° for 20 min., brought to the boil, left in the ice box for 1 hr. and centrifuged at 12,000 r.p.m. for 20 min. 3·0 ml. of supernatant were made up to 4·5 ml. Samples from tubes 1, 2, 3 and 4 were examined in the "Spekker" spectrophotometer using a sample from tube 5 as control.

Table 6. *Reduction of coenzyme I*

	Additions to tubes (ml.)				
	1	2	3	4	5
Enzyme	1·0	1·0	1·0	—	1·0
Enzyme boiled	—	—	—	1·0	—
Phosphate *M*/15 *p*H 7·4	—	1·0	1·0	1·0	1·0
Coenzyme I 5 mg./ml. 14% pure	1·0	1·0	1·0	1·0	—
Hexosediphosphate *M*/20	1·0	1·0	1·0	1·0	1·0
Arsenate *M*/10	—	—	0·5	0·5	0·5
Water	2·0	1·0	0·5	0·5	1·5
Log I_0/I at 345 mμ.	0·0	0·1	1·1	0·0	—

Oxidation of reduced coenzyme I. Reduced coenzyme I was prepared using lactic dehydrogenase of pig's heart. The heart was freed from fat, ground in a Latapie mincer and left on ice with 3 vol. of water for 30 min. The suspension was strained through muslin and centrifuged. The supernatant was made half saturated with ammonium sulphate and the resulting precipitate washed with half saturated ammonium sulphate. This precipitate was dissolved in water and dialysed for 16 hr. against running water. Insoluble material was removed by centrifuging.

1·0 ml. lactic enzyme, 5·0 mg. coenzyme I (14% pure), 0·3 ml. 2*M* cyanide, 0·3 ml. 2*M* sodium lactate and 1·4 ml. *M*/10 phosphate buffer *p*H 7·2 were incubated in a Thunberg tube for 30 min. Contents were boiled, 2·0 ml. water added and the mixture filtered through Whatman No. 40 paper. Filtrate was diluted 1 in 1 and examined spectrophotometrically. Log I_0/I at 345 mμ. = 1·3.

3·0 ml. reduced coenzyme I prepared as above, 1·0 ml. bacterial enzyme and 0·5 ml. *M*/10 phosphoglycerate were incubated anaerobically for 30 min. Contents were boiled, diluted with 1·5 ml. water and centrifuged. The supernatant examined spectrophotometrically gave log I_0/I at 345 mμ. 0·2. Control with boiled enzyme, log I_0/I 1·2.

Thus if 1:3-diphosphoglyceric acid is the first product of the oxidation, then the juice contains the system which phosphorylates 3-phosphoglyceric acid. According to Warburg & Christian [1939] neither 3-phosphoglyceric acid, 2:3-diphosphoglyceric acid nor phosphopyruvic acid affects the equilibrium between oxidized and reduced coenzyme I in the case of the purified yeast enzyme.

VI. *Kinetics*

Figs. 2, 3, 4 and 5 indicate how the reaction with molecular O_2 varies with the concentrations of four of the components of the system: coenzyme I (Fig. 2),

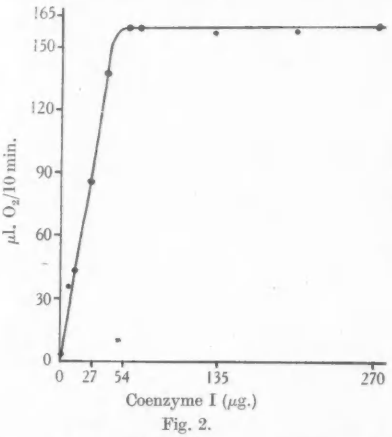

Fig. 2.

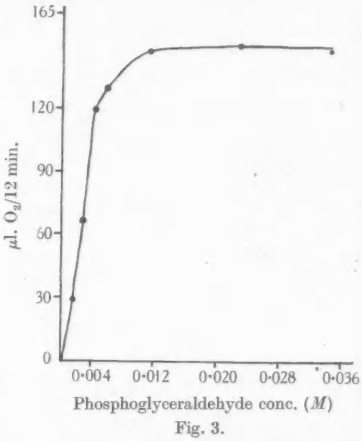

Fig. 3.

Fig. 2. Effect of concentration of coenzyme I. Manometers containing 1·0 ml. enzyme, 0·32 ml. *M*/10 arsenate, 0·1 ml. *M* NaF, 0·5 ml. 0·5 % methylene blue, 0·4 % ml. *M*/22 hexosediphosphate. Total volume 3·2 ml.

Fig. 3. Effect of concentration of substrate. Manometers containing 1·0 ml. enzyme, 0·2 ml. *M*/2 arsenate, 1 mg. coenzyme I, 0·5 ml. 0·5 % methylene blue, 0·1 ml. *M* NaF. Total volume 3·2 ml.

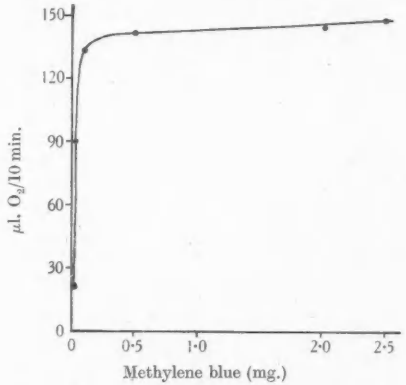

Fig. 4. Effect of concentration of carrier. Manometers containing 1·0 ml. enzyme, 0·32 ml. *M*/10 arsenate, 0·4 ml. *M*/22 hexosediphosphate, 0·1 ml. *M* NaF, and 1 mg. coenzyme I. Total volume 3·2 ml.

substrate (Fig. 3), carrier (Fig. 4) and arsenate (Fig. 5). Over relatively small ranges of concentration the rate of oxidation was proportional to the concentration. In the case of arsenate larger concentrations were found to be inhibitory.

For the anaerobic reaction in Thunberg tubes *p*H 7·4 was found to be optimum reaction. The behaviour of the uptake of molecular O_2 within the phosphate buffer range is shown in Fig. 6.

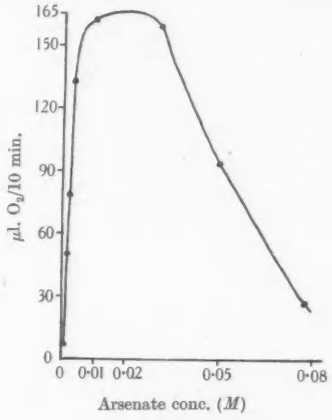

Fig. 5.

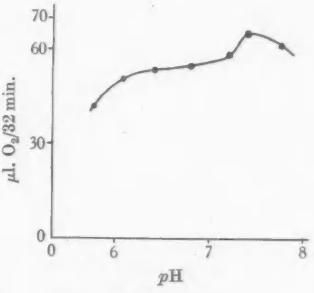

Fig. 6.

Fig. 5. Effect of concentration of arsenate. Manometers containing 1·0 ml. enzyme, 1 mg. coenzyme I, 0.5 ml. 0.5 % methylene blue, 0·4 ml. *M*/22 hexosediphosphate and 0·1 ml. *M* NaF. Total volume 3·2 ml.

Fig. 6. Effect of *p*H. Manometers containing 0·5 ml. enzyme, 1·2 ml. *M*/5 phosphate buffer, 0·4 ml. *M*/22 hexosediphosphate, 0·5 ml. 0·5 % methylene blue, 1 mg. coenzyme I and NaF. Total volume 3·2 ml.

Effect of iodoacetate. Inhibition of oxidation of phosphoglyceraldehyde by iodoacetate has been observed in yeast [Dixon, 1937] and in muscle [Green *et al.* 1937]. As the inhibitor is competing with the substrate the effect can be enhanced if the enzyme and inhibitor are incubated together first. The bacterial enzyme also behaves in this way (Table 7).

Table 7. *Effect of iodoacetate*

Figures refer to % inhibition

	M/10,000	*M*/2000	*M*/1000	*M*/100
No incubation	0	27	57	100
Incubation for 10 min.	0	69	75	100

VII. *Oxidation of phosphoglyceraldehyde by oxaloacetate*

The enzyme preparation contained also malic dehydrogenase, so that a coenzyme-linked reaction between phosphoglyceraldehyde and oxaloacetate was possible:

Phosphoglyceraldehyde + oxaloacetate → phosphoglycerate + malate.

The reaction was followed by the liberation of CO_2 from $NaHCO_3$-CO_2 buffer, an acid being produced from an aldehyde. Table 8 and Fig. 7 demonstrate the occurrence of this reaction and its components.

Table 8. *Oxidation of phosphoglyceraldehyde by oxaloacetate. Addition to manometers (ml.)*

Enzyme	1·0	1·0	1·0	1·0	1·0	1·0
$NaHCO_3$ *M*/10	0·8	0·8	0·8	0·8	0·8	0·8
NaF *M*	0·1	0·1	0·1	0·1	0·1	0·1
Hexosediphosphate *M*/20	0·4	0·4	0·4	—	0·4	0·4
Oxaloacetate 12·5 mg./ml.	0·4	0·4	0·4	0·4	0·4	—
Coenzyme I 5 mg./ml. 27% pure	0·2	0·2	0·2	0·2	—	0·2
Arsenate *M*/10	—	—	0·32	0·32	0·32	0·32
Phosphate *M*/10	—	0·32	—	—	—	—
Water	0·3	0·02	0·02	0·38	0·12	0·32
μl. CO_2 in 10 min.	33·5	38·5	421	16	19·5	45·5

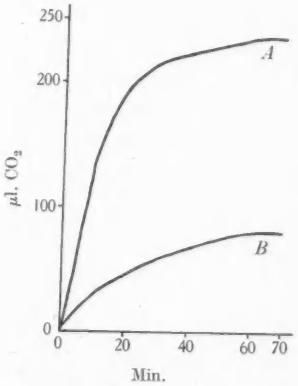

Fig. 7. Effect of arsenate (*A*) and phosphate (*B*) on the oxidation of phosphoglyceraldehyde by oxaloacetic acid. Manometers contained 1·0 ml. enzyme 0·8 ml. *M*/10 $NaHCO_3$, 0·2 ml. *M*/22 hexósediphosphate, 1 mg. coenzyme I, 0·1 ml. *M* NaF and 2·5 mg. oxaloacetic acid. Total volume 3·2 ml. Gas space N_2 5% CO_2.

This oxido-reduction is also inhibited by iodoacetate as shown in Table 9. The enzyme used in this experiment had not been dialysed free of phosphate, and arsenate was not added.

Table 9. *Effect of iodoacetate on oxido-reduction*

Manometers contained: 1·0 ml. enzyme, 0·3 ml. hexosediphosphate 0·057 *M*, 0·8 ml. *M*/10 $NaHCO_3$, 1 mg. coenzyme I 6·8% pure, 2·2 mg. oxaloacetic acid. Total volume 3·2 ml. in Barcroft manometers. Iodoacetate was added from Keilin cups.

	μl. CO_2/16 min.
Complete system	208
,, ,, − oxaloacetate	21
,, ,, + *M*/100 iodoacetate	15
,, ,, ,, ,, − oxaloacetate	20

VIII. *Dismutation of phosphoglyceraldehyde*

The enzyme preparation dismuted phosphoglyceraldehyde (Table 10). Presumably the reaction is:

2 Phosphoglyceraldehyde → phosphoglyceric acid + α-glycerophosphate.

Comparison of columns 3 and 6 in Table 8 shows that under identical conditions the rate of dismutation is about one-tenth the rate of oxido-reduction when oxaloacetic acid is the acceptor [Green *et al.* 1937].

Table 10. *Dismutation of phosphoglyceraldehyde by enzyme. Additions to manometers (ml.)*

Enzyme	1·0	1·0	1·0	1·0
$NaHCO_3$ *M*/10	0·8	0·8	0·8	0·8
NaF *M*	0·1	0·1	0·1	0·1
Hexosediphosphate *M*/20	0·4	0·4	0·4	—
Coenzyme I 5 mg./ml. 27% pure	0·2	0·2	—	0·2
Arsenate *M*/10	0·32	—	0·32	0·32
Phosphate *M*/10	—	0·32	—	—
Water	0·38	0·38	0·58	0·78
μl. CO_2 in 30 min.	95	27	2	10

IX. *Product of oxidation*

In § IV it was shown that the manometric data corresponded to an uptake of one atom of oxygen per molecule of phosphoglyceraldehyde, i.e. an oxidation to phosphoglyceric acid. In an experiment similar to that in Table 8 it was shown that difficultly hydrolysable P accumulated in the presence of NaF. Phosphate not hydrolysed by 3 hr. boiling with *N* HCl was considered as phosphoglyceric acid. As arsenate was present the method of Pett [1933] was used in the estimation of phosphate. Of the acid formed 85% could be accounted for as phosphoglyceric acid. In the absence of fluoride no difficultly hydrolysable phosphate accumulated.

Discussion

Recently Wiggert *et al.* [1940] have described the preparation of a cell-free juice from certain bacteria by grinding the cells with finely powdered pyrex glass. Their preparation from *Escherichia coli* when shaken with glucose and hexosediphosphate in $NaHCO_3$-CO_2 buffer produced acid, the amount of which was increased by addition of boiled yeast extract. Acetone preparations of *Bact. coli* carry out this reaction with catalytic amounts of hexosediphosphate and in the presence of NaF phosphoglyceric acid can be demonstrated as a reaction product [Endo, 1938].

It is now established that *Bact. coli* contains two enzymes similar to if not identical with the reducing and the oxidizing enzymes of yeast fermentation, alcohol and triosephosphate dehydrogenases. Endo [1938] concluded that the same coupled reactions and the same phosphorylated intermediate products occurred in *Bact. coli* fermentation as in yeast fermentation. The oxidizing enzyme probably plays the same part in the two processes; i.e. the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. The origin of acetaldehyde to act as substrate for the reducing enzyme is not clear. However, according to Tikka [1935] the alcohol resulting from the fermentation arises from α-glycerophosphate.

Pyruvic acid is the parent substance of alcohol in yeast fermentation. From the results of unpublished work by the author with cell-free preparations of *Bact. coli* it would seem that this organism contains no carboxylase but oxidizes pyruvic acid to acetic acid and CO_2. Inorganic phosphate, cocarboxylase and a heavy metal are involved in the reaction *in vitro*.

SUMMARY

1. A cell-free extract of *Bact. coli commune* containing zymohexase and phosphoglyceraldehyde dehydrogenase has been made.

2. As coenzyme I and either inorganic phosphate or arsenate are required for the oxidation of phosphoglyceraldehyde the following course of reaction is assumed in the presence of fluoride:

3-phosphoglyceraldehyde → 1:3-diphosphoglyceraldehyde →
1:3-diphosphoglyceric acid → 3-phosphoglyceric acid.

3. The reaction is shown to be reversible with respect to the reduction and reoxidation of coenzyme I.

4. The kinetics of this reaction where molecular oxygen is the oxidant have been investigated.

5. Phosphoglyceraldehyde dehydrogenase is inhibited by iodoacetate.

6. As malic dehydrogenase is also present in the enzyme preparation, the phosphoglyceraldehyde can be oxidized in a coenzyme-linked reaction with oxaloacetic acid.

7. The enzyme preparation will dismute phosphoglyceraldehyde under suitable conditions.

8. The product of oxidation was not isolated but evidence is given that phosphoglyceric acid is the main or only product.

The author is very grateful to Dr D. E. Green for his advice in this work, also to the Hebden Medical Fund of the University of Sydney for an assistance grant. The crushing mill used in this investigation was provided by a grant to this laboratory by the Medical Research Council.

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164. BIOLOGICAL SYNTHESIS OF OXALOACETIC ACID FROM PYRUVIC ACID AND CARBON DIOXIDE

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(*Received 30 September 1940*)

It was recently shown that pigeon liver synthesizes α-ketoglutarate from added pyruvate [Evans, 1940]. Experiments which were undertaken to elucidate the mechanism of this synthesis suggest that the primary step is a synthesis of oxaloacetate from pyruvate and carbon dioxide:

(1) $CO_2 + CH_3.CO.COOH \longrightarrow COOH.CH_2.CO.COOH$,

or

$$H_2CO_3 + CH_3.CO.COOH \xrightarrow{-H_2O} COOH.CH_2.CO.COOH$$

or

$$HCO_3' + CH_3.CO.COO' \xrightarrow{-H_2O} COO'.CH_2.CO.COO'.$$

The oxaloacetate formed in this way is converted into citrate and α-ketoglutarate through the citric acid cycle, as previously described:

(2) 2 Oxaloacetate + pyruvate → citrate + "malate" + CO_2

and

(3) Citrate + oxaloacetate → α-ketoglutarate + "malate" + CO_2.

The net effect of 1, 2 and 3 is a synthesis of α-ketoglutarate (and malate) from pyruvate:

(4) 4 Pyruvate + CO_2 = α-ketoglutarate + 2 "malate"[1].

Experiments on tissues of vitamin B_1-deficient pigeons suggest that reaction 1 is the stage at which the vitamin enters the metabolism of pyruvate in animal tissues. This result removes one of the difficulties confronting the theory of the vitamin effect. From Peters' work it was known that the oxidation of pyruvate in animal tissues depends on the presence of vitamin B_1. In yeast the vitamin is required for the non-oxidative decarboxylation of pyruvate. It has been difficult to understand how one and the same catalyst could catalyse an oxidation in one cell and a non-oxidative process in another. It now appears that the vitamin is not directly concerned with the oxidation of pyruvate in animal tissues, but with a reaction preparatory to the oxidation, the "carboxylation" of pyruvate. This reaction is analogous to the decarboxylation of pyruvate in yeast (as can be demonstrated in model experiments) and there is no theoretical difficulty in attributing these two analogous reactions to the same catalyst.

The occurrence of reaction 1 has also been suggested by recent observations in the field of bacterial metabolism. Wood & Werkman [1938; 1940, 1], studying the metabolism of propionic acid bacteria, discovered that these organisms utilize inorganic CO_2, and form succinate in quantities equivalent to the CO_2 used. Elsden [1938] showed that the formation of succinate in *B. coli* depends on the concentration of CO_2. Commenting on their observations, Wood & Werkman

[1] "Malate" stands for the equilibrium mixture of fumarate and *l*(−)malate.

wrote [1938] "Pyruvic acid...may be the point of entry for CO_2" and later [1940, 1]: "A possible mechanism accounting for CO_2 utilization as well as succinic acid formation involves the addition of CO_2 to pyruvic acid to form oxaloacetic acid, followed...by the reduction to succinic acid." Experiments on *B. coli*, reported in this paper, fully support this hypothesis and the inference is that the synthesis of oxaloacetate from pyruvate and CO_2 is a general metabolic process which occurs in widely different organisms.

I. *Experiments on pigeon liver*

1. *Methods.* Pigeon liver was minced and suspended in saline media as described by Evans [1940] and Krebs & Eggleston [1940]. The suspensions were incubated under varying conditions at 40°. The following media were used:

(1) "Phosphate saline, *p*H 7·4", prepared as described by Krebs & Eggleston [1940].

(2) "Phosphate saline, *p*H 7·1", prepared in the same way as medium (1), except that the phosphate buffer used was made by diluting 33·3 ml. *N* HCl and 17·8 g. Na_2HPO_4, $2H_2O$ to 1 l.

(3) "Phosphate-bicarbonate-saline, *p*H 7·4", prepared by addition of 20 ml. 1·3 % $NaHCO_3$ to 100 ml. medium (1); *p*H is approximately 7·4 when the medium is in equilibrium with a gas mixture containing 5 % CO_2.

(4) "Phosphate-bicarbonate-saline, *p*H 7·1", prepared by addition of 10 ml. 1·3 % $NaHCO_3$ to 100 ml. medium (2); *p*H is approximately 7·1 when the medium is in equilibrium with a gas mixture containing 5 % CO_2.

Since the tissue contains and produces considerable amounts of acid substances, *p*H of the tissue suspension is always lower than that of the medium used. The metabolic reactions under discussion are however little affected by a change of acidity between *p*H 6·8 and 7·4 and no attention was therefore paid to the accurate adjustment or determination of *p*H.

The analytical methods were the same as in the previous papers from this laboratory [see Krebs & Eggleston, 1940; Krebs, 1940; Krebs, Smyth & Evans, 1940; Krebs, Eggleston, Kleinzeller & Smyth, 1940].

2. *Effect of CO_2 on pyruvate utilization.* Two suspensions of the same liver were prepared, one in phosphate saline, and the other in phosphate-bicarbonate-saline of the same *p*H. Of each suspension 20 ml. were poured into a 50 ml. measuring cylinder and placed in a water bath at 40°. O_2 was passed at a rapid rate through the first suspension, O_2 with 5 % CO_2 through the second. The rate of gas flow was approximately 100 ml. per min. At the end of the incubation period 4 ml. 2*N* HCl were added to the suspensions whilst they were still in the water bath. After about 2 min. they were removed from the bath and pyruvate, citrate, α-ketoglutarate, succinate and fumarate + malate were determined in aliquots of the suspension. The results are shown in Table 1.

In the presence of bicarbonate and CO_2 the rate of pyruvate utilization is 37–94 % above that of the controls. There is also a large increase in the formation of α-ketoglutarate (115–170 %) of citrate (160–560 %) and of fumarate + malate (125–400 %).

These effects of CO_2 and bicarbonate, taken alone, are by no means definite proof of the participation of these substances in the chemical processes of the tissue. To explain the effects, it might be argued that CO_2 and bicarbonate are essential constituents of a "balanced medium" and that their action is similar to that of the other inorganic substances. Against this argument it can be pointed out that unlike the other inorganic substances, CO_2 and bicarbonate

Table 1. *Effect of CO_2 pressure on metabolism of pyruvate in pigeon liver*

All data refer to 10 ml. suspension; the quantities of metabolites are expressed in μl.

Exp. no.	1		2		3	
Suspension used:	1 part liver + 13 parts medium		1 part liver + 13 parts medium		1 part liver + 9 parts medium	
Medium	Phosphate saline *p*H 7·1	Phosphate bicarbonate saline *p*H 7·1	Phosphate saline *p*H 7·1	Phosphate bicarbonate saline *p*H 7·1	Phosphate saline *p*H 7·4	Phosphate bicarbonate saline *p*H 7·4
Gas	O_2	5% CO_2 in O_2	O_2	5% CO_2 in O_2	O_2	5% CO_2 in O_2
Period of incubation (min.)	30	30	45	45	30	30
Initial pyruvate	9220	9220	8600	8600	8940	8940
Final pyruvate	7100	6320	6250	4050	7230	5660
Pyruvate used	2120	2900	2350	4550	1710	3280
Succinate formed	26	72	45	85	35	52
α-Ketoglutarate formed	277	680	435	938	285	763
Fumarate + *l*(−)malate formed	269	922	191	426	44	217
Citrate formed	46	120	46	276	40	263

have no general effect on metabolic processes in minced liver. As far as is known their effects on pyruvate utilization are specific; a specific chemical hypothesis, such as reaction 1, offers therefore a more satisfactory explanation.

3. *Anaerobic formation of α-ketoglutarate, citrate, fumarate and malate from pyruvate.* It was already mentioned by Evans [1940] that α-ketoglutarate can be formed from pyruvate under anaerobic conditions. In Evans's experiments phosphate saline was used as a medium and under these conditions the formation of α-ketoglutarate varied in magnitude and did not regularly occur. We find that the formation of α-ketoglutarate is larger and more consistent when "phosphate-bicarbonate-saline" is used as a medium, and that fumarate and malate are formed in considerable quantities together with α-ketoglutarate. Small amounts of citrate and of succinate are also formed (Table 2).

Table 2. *Formation of succinate, α-ketoglutarate, fumarate + malate and citrate from pyruvate under anaerobic conditions*

40°; 5% CO_2 in N_2; period of incubation 60 min.

	Exp. 1		Exp. 2	
Suspension used	1 part liver, 8 parts phosphate-bicarbonate saline, *p*H 7·4		1 part liver, 9 parts phosphate-bicarbonate saline, *p*H 7·4	
Quantity of suspension	3 ml.		4 ml.	
Substrate	— (Control)	0·02 *M* pyruvate	— (Control)	0·1 *M* pyruvate
μl. Pyruvate used	—	1157	—	890
μl. Succinate formed	0	52	20	45
μl. α-Ketoglutarate	12	142	60	131
μl. Fumarate + malate	26	216	100	334
μl. Citrate	—	—	6	12

The following facts are relevant with regard to the explanation of the simultaneous formation of citrate, α-ketoglutarate, succinate, fumarate and malate:

(*a*) The substances enumerated are interconvertible, through the citric acid cycle, if the conditions are aerobic, but α-ketoglutarate and malate are not interconvertible if the conditions are anaerobic. Citrate can anaerobically be converted into α-ketoglutarate only if oxaloacetate is present [Krebs, 1940]. Succinate can arise anaerobically from both α-ketoglutarate and fumarate.

(*b*) Anaerobically oxaloacetate yields citrate, α-ketoglutarate, succinate, fumarate and malate [Krebs, Eggleston, Kleinzeller & Smyth, 1940], i.e. the same substances which are formed from pyruvate in the presence of CO_2 and bicarbonate (Table 2). The proportions in which the substances appear are similar with oxaloacetate and with pyruvate; the rate of reaction is more rapid with oxaloacetate.

The results recorded in Table 2 may be explained in two different ways. Either it may be assumed that pyruvate is converted into oxaloacetate which in turn reacts to form the substances found as end-products. Or it may be assumed that there are three different and independent reactions leading from pyruvate: (1) to citrate, (2) to α-ketoglutarate, (3) to fumarate and malate. This second alternative is improbable and unsupported by experimental evidence. The first explanation, on the other hand, is supported by independent evidence.

4. *Aerobic formation of citrate, α-ketoglutarate, fumarate and malate from pyruvate.* Since citrate, α-ketoglutarate, fumarate and malate are aerobically interconvertible, as was pointed out in the previous paragraph, the simultaneous accumulation of all the substances is not unexpected when O_2 is present. Yet it can be shown by quantitative determination of all the reactants, including O_2, that under certain conditions the end-products cannot be formed by oxidative interconversion through the citric acid cycle. The O_2 uptake is too small to account for this mechanism. This holds if the pyruvate concentration is high (0·1 *M*) and the experimental period short (20 min.). Under such conditions reactions different from those forming the citric acid cycle prevail.

An experiment is shown in Table 3. Ten conical manometer cups were used, 5 of which had two side arms. The first 5 cups contained 3 ml. liver suspension in the main compartment and 0·5 ml. 2 *N* HCl in the side arm. The remaining 5 cups had, in addition, 1 ml. 0·4 *M* pyruvate in the second side arm. The gas space was filled with N_2. The cups were shaken at 40° and after 10 min. the acid

Table 3. *Formation of citrate, α-ketoglutarate, succinate, fumarate and malate, and acetoacetate, from pyruvate under aerobic conditions*

Suspension: 1 part liver, 6·5 parts phosphate bicarbonate saline *p*H 7·4; 2·5% CO_2 in O_2. The pigeon was starved for 48 hr. The data refer to 3 ml. suspension.

	Zero value μl.	After 20 min. incubation μl.	Change μl.
Pyruvate	8880	7150	−1630
Citrate*	0	135	+ 135
α-Ketoglutarate	87	376	+ 289
Succinate	0	73	+ 73
Fumarate + malate	134	351	+ 217
Acetoacetate	7	52	+ 45
O_2 used	—	760	− 760
CO_2 formed (total)	—	877	+ 877

* Including *iso*citrate and *cis*-aconitate.

was added to the tissue in the first 5 cups to stop the metabolism. The determination in these cups provided the zero values. At the same time the pyruvate was added to the liver suspension in the other cups and shaking was continued for 20 min. The acid was then added from the second side arm. The contents of separate cups were used for the determination of succinate, α-ketoglutarate, fumarate + malate and of acetoacetate. The fifth cup was used for the determination of pyruvate and of citrate. In a parallel experiment the O_2 uptake and the CO_2 output were measured by the Dickens-Simer [1931] method. Since in this method no substrate can be mixed with the tissue after the closing of the manometer, pyruvate had to be added before the manometer was placed in the bath. Thus the period of measurement, beginning 15 min. later, did not exactly coincide with that of the first 10 cups. There are, however, good reasons for assuming that the rate of O_2 uptake is linear and that it is justifiable to extrapolate accordingly for the period preceding the measurements.

With the exception of acetoacetate the products found in this experiment are the same as those formed under anaerobic conditions. The quantities, however, are considerably larger, especially in the case of citrate. Moreover, these larger quantities are formed in a much shorter period of incubation (20 min., against 60 min. in the aerobic experiments) and it follows that the rates of reaction are much higher in the presence of O_2.

Before discussing the interpretation of these results it is necessary to consider the assumptions which may be made to explain the utilization of pyruvate. If it is assumed that a condensation of 2 molecules of pyruvate of the type discussed by Toeniessen & Brinkmann [1930; see also Krebs, 1936] occurs, the formation of 1 molecule of α-ketoglutarate requires 2 molecules of pyruvate and 1 molecule of O_2. If succinate, fumarate, malate and citrate arise from α-ketoglutarate by way of the citric acid cycle, the relations between the products formed and pyruvate and O_2 usages are as shown in the following list:

Substance formed from pyruvate	Molecules of pyruvate required	Molecules of O_2 required
α-Ketoglutarate	2	1
Succinate	2	1½
Fumarate + malate	2	2
Citrate	3	3
Acetoacetate	2	1

This hypothesis, applied to the experiment of Table 3, would postulate an O_2 uptake of 1283 μl., but only 760 μl. were absorbed. The hypothesis must therefore be discarded. Other schemes involving the primary formation of succinate from acetate [see Weil-Malherbe, 1937] are still less satisfactory. An alternative explanation which is in accordance with the facts is offered by reactions 1, 2 and 3. According to this scheme no O_2 is required for the formation of citrate, α-ketoglutarate and fumarate and the anaerobic formation of these substances (Table 2) demonstrates that O_2 is in fact not essential. If O_2 increases the rate of reaction—as it does—its effect on reaction 1 must be indirect, for instance through the supply of energy. Reaction 1 being endothermic requires energy; the supply of energy is comparatively small under anaerobic conditions and is increased when O_2 is available.

5. *Utilization of CO_2.* According to 4, CO_2 disappears when reactions 1, 2 and 3 occur. It has not been possible, however, to demonstrate the disappearance of CO_2 in the presence of pyruvate and this is due to the fact that 4 is not the only reaction occurring under the conditions of the experiments. At least two other

reactions in which CO_2 is formed take place, viz. the formation of acetoacetic acid and the dismutation of pyruvate:

(5) 2 Pyruvate + O_2 → acetoacetate + 2 CO_2,
(6) 2 Pyruvate + H_2O → lactate + acetate + CO_2.

The disappearance of CO_2 in 4 is counterbalanced by the production of CO_2 in 5 and 6. At present there seems to be little prospect of separating 4 from other reactions; perhaps it will be possible to demonstrate the utilization of CO_2 conclusively by the use of carbon isotopes.[1]

6. *The role of vitamin B_1.* It is known from the work of Peters and his school [Peters & Sinclair, 1933; Peters & Thompson, 1934; Thompson, 1934; Peters, 1936, 1, 2; McGowan, 1937; McGowan & Peters, 1937; Peters, 1937; Ochoa & Peters, 1938; Banga *et al.* 1939; Long & Peters, 1939; Peters, 1940, 1, 2] that vitamin B_1 is concerned with the utilization of pyruvate in pigeon brain and in pigeon kidney. The nature of the chemical reaction catalysed by the vitamin in animal tissues has been much discussed [see Peters and his school; Lipmann, 1937, 1, 2; Quastel & Webley, 1939; Weil-Malherbe, 1940], but the problem must still be regarded as unsolved. On the other hand, the reaction effected by the vitamin in yeast is known [Lohmann & Schuster, 1937]: the vitamin, in the form of its pyrophosphate ester, catalyses the conversion of pyruvic acid into acetaldehyde:

$$(7)\quad CH_3.CO.COOH \xrightarrow{\text{vitamin pyrophosphate}} CH_3.CHO + CO_2.$$

This reaction does not occur in animal tissues. Hitherto no reaction which can be regarded as analogous to 7 was known to take place in animal tissues and the vitamin effects in yeast and in animal tissues therefore appeared to be due to two different mechanisms, one oxidative and the other non-oxidative. In view of the high degree of specificity generally shown by biological catalysts the assumption of such a dual function is somewhat unsatisfactory.

The new reaction 1 resembles 7 more closely than any other reaction known to occur in animal tissues, and in view of this it seemed probable to us that reaction 1 might be the reaction catalysed by the vitamin in animal tissues.

To test this hypothesis pigeons were fed on polished rice until the symptoms of severe beriberi appeared (head retraction, paralysis or weakness of legs and wings). The removal of pyruvate from liver suspensions was then measured under conditions in which reaction 1 is assumed to be the chief metabolic reaction responsible for the removal of pyruvate, i.e. at high concentrations of pyruvate and of CO_2, and in the presence of O_2. Further details of the conditions are given in Table 4.

Under the conditions of these experiments, the rate of pyruvate removal was about 2000–3000 μl. per hr. (Q_{pyruvate} about -35) when normal pigeons were used. The rate was found to be greatly reduced in vitamin-deficient animals and the degree of the reduction was greater the severer the symptoms. In Exp. 1, where the symptoms were severest, the rate was reduced to 675 μl. per hr., and in Exp. 2 where symptoms of deficiency were less pronounced, the rate was 1790 μl. per hr. Addition of small quantities of vitamin restore the rate to normal level. The effect of the vitamin is very striking: in Exp. 1, 0·6 μg. per ml. liver suspension increased the pyruvate usage from 675 to 2100 μl., i.e. more than 300 %. The quantities of vitamin required are similar to those in brain.

When the same experiment was carried out with muscle tissue of vitamin-deficient pigeons, no significant vitamin effect was observed (Table 5). Both

[1] See note at the end of this paper.

Table 4. *Effect of vitamin B_1 on pyruvate utilization in pigeon liver*

Minced pigeon liver suspended in 6·5 parts phosphate-bicarbonate saline, *p*H 7·4; 3 ml. of this suspension mixed with 1 ml. approx. 0·2 *M* pyruvate; 5% CO_2 in O_2; period of incubation 60 min.; duplicates in several experiments.

No.	State of pigeon	Quantity of aneurin (Roche Products) added per ml. suspension μg.	μl. initial pyruvate in suspension (4 ml.)	μl. final pyruvate	μl. pyruvate utilized	Increase due to vitamin (average) %
1	Fed 22 days on polished rice. Paralysis of legs and wings, head retraction	0	4360	3690	670	—
		0	4360	3680	680	
		0·625	4360	2250	2110	211
		0·625	4360	2270	2090	
		6·25	4360	2365	1995	178
		6·25	4360	2430	1930	
		62·5	4360	2285	1975	198
		62·5	4360	2315	2045	
2	Fed 25 days on polished rice, weakness of legs and wings, no head retraction	0	4440	2650	1790	
		0·2	4440	2370	2070	16
		1·0	4440	2065	2375	33
3	Fed 24 days on polished rice. Severe paralysis of wings and legs, no head retractions, no convulsions	0	4350	2865	1485	
		0	4350	3210	1140	
		0·8	4350	1620	2730	99
		0·8	4350	1865	2475	
4	Fed 35 days on polished rice. Severe paralysis of legs and wings, no head retraction, no convulsions	0	4300	3255	1045	164
		0·8	4300	1540	2760	
5	Normal, well fed	0	4440	1955	2395	
6	Normal (starved 48 hr.)	0	4440	2685	1755	
		20	4440	2780	1660	
7	Normal, well fed	0	4440	980	3460	
8	Normal, well fed	0	4500	830	3670	

Table 5. *Effect of vitamin B_1 in muscle and liver of the same pigeon*

Experimental conditions as stated in Table 4

Tissue used	Quantity of vitamin added (μg. to 4 ml.)	μl. pyruvate used
Breast muscle	0	440
,,	0·8	470
Liver	0	1540
,,	0·8	2760

liver and muscle can metabolize pyruvate through the citric acid cycle [Krebs & Eggleston, 1940; Evans, 1940], and if the vitamin played a part in one of the reactions of this cycle an effect would be expected in both tissues. The absence of the effect from muscle suggests that the vitamin takes part in a reaction of pyruvate which occurs in liver, but is of no major importance in muscle. So far two reactions to which this applies are known: reaction 1 and the synthesis of ketone bodies (reaction 5), and since there is no reason to assume that other reactions are of major importance for pyruvate removal in pigeon liver, we come to the conclusion that one of the reactions 1 and 5—or both—require the vitamin.

To examine this question experimentally, the effect of the vitamin on acetoacetate formation was measured in vitamin-deficient liver (Table 6). The acetoacetate formation was found to be somewhat reduced in the B_1-deficient

Table 6. *Effect of vitamin B_1 on the acetoacetate production in pigeon liver*

Experimental conditions as stated in Table 4; acetoacetate was determined according to Edson [1935].

No.	Condition of pigeon	Quantity of vitamin added μg. per ml. suspension	μl. pyruvate added	μl. pyruvate used	μl. acetoacetate found
1	Fed 24 days on rice. Severe paralysis of legs and wings	0	4350	1140	63
		0·8	4350	2475	224
2	Fed 35 days on rice. Severe paralysis of legs and wings	0	4300	1045	27
		0·8	4300	2760	58
3	Normal, well fed	0	4500	3670	210
	,,	25	4500	3520	246
4	,,	0	4440	3460	87
5	,,	0	4440	2395	128

animals (average 45 μl. against 142 μl. in normal liver). Addition of the vitamin restored the acetoacetate formation to the normal level. Compared with the absolute increase in pyruvate usage, however, the increase in acetoacetate formation is small. In Exp. 1 the acetoacetate formation accounts for 24 %, in Exp. 2 for only 3·6 % of the pyruvate usage caused by the vitamin (calculated on the basis of scheme 5).

It might be concluded from these experiments that the vitamin takes part in both the synthesis of oxaloacetate and the synthesis of acetoacetic acid and such a hypothesis is not unattractive considering that both reactions are probably analogous in their mechanism: the most likely mechanism of acetoacetate formation involves the synthesis of acetopyruvate [Krebs & Johnson, 1937]:

$$(8)\quad CH_3.COOH + CH_3.CO.COOH \xrightarrow{-H_2O} CH_3.CO.CH_2.CO.COOH.$$

The analogy with reaction 1 is obvious if the latter is written in the following form:

$$HO.COOH + CH_3.CO.COOH \xrightarrow{-H_2O} HO.CO.CH_2.CO.COOH.$$

However, the effect of the vitamin on acetoacetate formation, as shown in Table 6, is not necessarily a direct one. The synthesis of ketone bodies may be linked up with other processes and indirectly dependent on reaction 1. It must therefore be left open whether the vitamin takes part in reaction 1 only, or in both 1 and 8.

The conclusions reached here are in accordance with the view of McGowan & Peters [1937] that in animal tissues vitamin B_1 is concerned specifically with pyruvic acid, and not with α-ketonic acids in general. They also agree with the statement of Sober *et al.* [1940] that the vitamin "is essential for the synthesis of endogenous citric acid from its precursors", since reaction 1 is an essential step in the synthesis of citric acid.

The new conception has the great advantage of offering analogous explanations for the actions of the vitamin in yeast and in animal tissues. In yeast, its active form catalyses the "decarboxylation" of pyruvate (reaction 7); in

animal tissues it catalyses the "carboxylation" of pyruvate (reaction 1). The close analogy of the two reactions can be strikingly demonstrated by a simple model experiment: aniline is well known to catalyse the decarboxylation of oxaloacetate (i.e. the reversal of reaction 1); we find that the same catalyst can decarboxylate pyruvate according to reaction 7. The rate of the second reaction is very low when the pyruvate concentration is below 0·01 *M* and the reaction was therefore previously overlooked. The rate is rapid at high concentrations of pyruvate as shown in Table 7. Thus one and the same catalyst, aniline, can decarboxylate α-ketonic acids as well as β-ketonic acids. The fact that reaction 1 is reversed in the model hardly detracts from its instructive value.

Table 7. *Decarboxylation of pyruvic acid in the presence of aniline*

25°. 4 ml. pyruvic acid solution, 0·5 ml. 50% citric acid, 0·5 ml. aniline citrate [see Edson, 1935].

Final concentration of pyruvic acid (*M*)	μl. CO_2 formed		
	10 min.	20 min.	30 min.
0·8	136	219	301
0·2	40	52	63
0·05	10	12	13
0·0125	0	0	0

II. *Synthesis of oxaloacetate in bacteria*

The recent literature on bacterial metabolism contains a number of observations which suggest that reaction 1 may also occur in propionic acid bacteria, *Bacterium coli commune*, and other bacteria.

Wood & Werkman showed that propionic acid bacteria utilize CO_2 and produce succinic acid in quantities equivalent to the amount of CO_2 used. This equivalence can be interpreted in two ways. In their first paper [1936] Wood & Werkman [see also Krebs, 1937] suggested that CO_2 might act as a hydrogen acceptor in one of the reactions leading to the formation of succinate. The equivalence would be accounted for, if the following reaction took place:

(9) $2CH_3.COOH - 2H + CO_2 \rightarrow COOH.CH_2.CH_2.COOH + HCOOH.$

Later Wood & Werkman [1940, 1] discussed a mechanism involving reaction 1 and a reduction of oxaloacetate to succinate:

(10) $\underset{\displaystyle CO.COOH}{\overset{\displaystyle CH_2}{|}} + CO_2 \rightarrow \underset{\displaystyle CO.COOH}{\overset{\displaystyle CH_2.COOH}{|}} \rightarrow \underset{\displaystyle CH(OH).COOH}{\overset{\displaystyle CH_2.COOH}{|}} \rightarrow \underset{\displaystyle CH.COOH}{\overset{\displaystyle CH.COOH}{\|}} \rightarrow \underset{\displaystyle CH_2.COOH}{\overset{\displaystyle CH_2.COOH}{|}}$

The experiments reported so far in the literature do not decide between these two alternatives.

For *B. coli* Elsden [1938] showed that the formation of succinate from pyruvate depends on the pressure of CO_2. This observation, again, can be explained by scheme 9 or scheme 10.

Studying the succinate formation from pyruvate in *B. coli* we have made a new observation which supports scheme 10. It was found that, together with succinate, fumarate and malate arise from pyruvate (Table 8). Since added succinate is not converted into fumarate under the conditions of the experiments (which were anaerobic), fumarate and malate cannot be formed through succinate and scheme 9 is thus ruled out as far as the formation of fumarate and malate is concerned. Although (theoretically) the formation of succinate through scheme 9 is not impossible, this scheme is now superfluous and we think it

Table 8. *Formation of succinate, fumarate and malate from pyruvate in* B. coli

15–20 mg. washed cells (dry weight) grown on broth agar [Krebs, 1937] with 2% glycerol, suspended in 4 ml. saline containing 0·05–0·1 *M* $NaHCO_3$; gas phase, CO_2; 40°. The CO_2 evolved comprises CO_2 liberated by acid from $NaHCO_3$ and CO_2 formed from pyruvate. The CO_2 output gives an indication of the extent to which the added pyruvate has been decomposed. One mol. of pyruvate yields approximately one mol. of CO_2.

Strain of *B. coli**	μl. pyruvate added	Period of incubation (min.)	μl. CO_2 evolved	μl. succinate	μl. fumarate + malate†
No. 3	4480	260	3500	398	650
No. 4705	4480	250	4720	350	575
No. 4705	2240	140	2210	187	870
No. 4833	2240	230	2250	127	325
No. 4854	2240	370	2195	158	86

* The strains were isolated from faeces and urine by Dr M. S. Spink and Dr D. Lewis in the Department of Bacteriology of this University.

† The ratio of fumarate/malate in the equilibrium mixture was found to be the same as in animal tissues [see Krebs, Smyth & Evans, 1940].

should therefore be discarded. For it seems highly improbable to us that there are in one cell two different mechanisms leading to the formation of a 4-carbon chain from pyruvate.

Scheme 10 postulates that oxaloacetate and fumarate are converted into succinate at least as rapidly as pyruvate. Data showing that this is the case have already been presented [Krebs, 1937].[1]

Discussion

1. *Appraisal of the evidence for reaction 1.* The evidence for reaction 1 is necessarily indirect, since the secondary reaction of oxaloacetate makes it impossible to demonstrate directly the disappearance of CO_2 and the formation of oxaloacetate. In animal tissues the secondary reactions are known to yield fumarate, malate, α-ketoglutarate, citrate, succinate and CO_2 [Krebs, Eggleston, Kleinzeller & Smyth, 1940] and these substances are therefore expected to be formed instead of oxaloacetate. This is the case, and since the proportions in which the various substances appear are of the expected order, the evidence for the synthesis of oxaloacetate may be regarded as satisfactory.

In *B. coli* the secondary reactions of oxaloacetate are somewhat different in detail though the underlying principle is the same. The common principle is the reduction of one part of the oxaloacetate at the expense of the oxidation of another part. The difference is in the nature of the oxidative and reductive reactions. In animal tissues the reduction leads mainly to malate and fumarate. In *B. coli* it proceeds further to the stage of succinate. The oxidative reactions in animal tissues are those formulated by the citric acid cycle. There is no citric acid cycle in *B. coli* and the details of the oxidative processes in this organism are not yet clear [see Krebs, 1937]. The main end-product of the oxidation is CO_2. Thus in the case of *B. coli* reaction 1 will result in an accumulation of succinate; conversely, the synthesis of succinate can be taken as evidence for reaction 1.

The failure to show directly the disappearance of CO_2 in animal tissues and in *B. coli* [see Krebs, 1937] is satisfactorily explained. On the other hand the

[1] Experiments on propionic acid bacteria supporting scheme 10 will be reported in a separate paper.

effect of CO_2 on pyruvate usage and on the formation of α-ketoglutarate, succinate and related compounds is direct evidence for the participation of CO_2 in the synthesis of these substances.

Although no single experiment represents conclusive evidence for reaction 1, we think that the force of the cumulative evidence leaves little doubt about the occurrence of the reaction.

2. *Analogous reactions* in vitro. It is of interest to note that the reaction of Grignard compounds with CO_2 is analogous to reaction 1, e.g.:

$$CH_3MgCl + CO_2 \xrightarrow{+H_2O} CH_3.COOH + Mg(OH)Cl.$$

In both cases CO_2 is added to the carbon chain and a carboxylic acid is formed. It may not be fortuitous that reaction 1, like the reaction of the Grignard compounds, depends on the presence of magnesium.

3. *Occurrence of reaction 1 in other tissues and organisms.* Reaction 1 appears to be of minor importance in skeletal and cardiac muscle, but it is very probable that it plays an important part in brain and in several other tissues. This remains to be investigated. It is also highly probable that the reaction occurs in those plants, moulds and bacteria [see Wood & Werkman, 1940, 2] in which oxaloacetate [see Virtanen & Laine, 1938], malate, succinate or citrate are formed.

4. *The role of vitamin B_1 in bacteria.* In *Staphylococci* and *Lactobacillus delbrucki* vitamin B_1 has been shown to catalyse the oxidative decarboxylation of pyruvate [Hills, 1938; Barron & Lyman, 1939; Lipmann, 1937, 2].

$$CH_3.CO.COOH \xrightarrow{+O} CH_3.COOH + CO_2.$$

In *Streptococcus haemolyticus* it appears to take part in the following reaction [Barron & Lyman, 1939].

$$CH_3.CO.COOH \xrightarrow{+H_2O} CH_3.COOH + H.COOH.$$

On first impressions it may be thought that the mechanism of the vitamin effect in these cases must be different from that proposed earlier in this paper. This is, however, not necessarily the case. There are several observations which suggest that oxaloacetate acts as a hydrogen carrier in the oxidation of pyruvate:

$$(11)\quad \begin{cases} \text{Pyruvate} + \text{oxaloacetate} + H_2O \rightarrow \text{acetate} + CO_2 + \text{malate}, \\ \text{Malate} + \text{pyruvate} \rightarrow \text{oxaloacetate} + \text{lactate}. \end{cases}$$

If this is true reaction 1 must occur in these organisms and the vitamin effect can be explained on the same basis as in animal tissues. Scheme 11 implies that the Szent-Györgyi cycle occurs in bacteria. Evidence in favour of this will be discussed in a subsequent paper.

5. *The mechanism of the biological succinic acid formation.* It is generally accepted that succinic acid can be synthesized by animal tissues, bacteria and moulds from compounds containing less than 4 carbon atoms. The mechanism of this synthesis has been discussed by many authors. The earliest hypothesis [Thunberg, 1920] suggested that the synthesis was brought about by condensation, coupled with dehydrogenation, of 2 molecules of acetic acid:

$$(12)\quad 2CH_3.COOH \xrightarrow{-2H} COOH.CH_2.CH_2.COOH.$$

Experiments designed to demonstrate a synthesis of succinate from added acetate failed and at the same time made it clear that pyruvate is a precursor

of succinate in animal tissues and in bacteria [Toeniessen & Brinkmann, 1930; Krebs & Johnson, 1937; Krebs, 1937; Weil-Malherbe, 1937; Elsden, 1938]. The above scheme was therefore modified. Toeniessen & Brinkmann [1930] assumed that 2 molecules of pyruvate condense to form $\alpha\alpha'$-diketo-adipic acid which in turn was believed to form succinate and 2 molecules of formate:

$$2COOH.CO.CH_3 \xrightarrow{-2H} COOH.CO.CH_2.CH_2.CO.COOH \rightarrow COOH.CH_2.CH_2.COOH + 2HCOOH$$

Krebs [1936] suggested that pyruvate and acetate may condense to form α-keto-glutarate and Weil-Malherbe [1937] thought that acetate may react according to (12) in the nascent state. These attempts to explain the fact that pyruvate can be converted into succinate were not supported by direct experimental evidence. It is now very probable that they are incorrect and that reaction (1) is the primary step by which the 4 carbon chain is synthesized from a shorter chain.

It is significant that the succinate formation from hexoses in *B. coli* also depends on the CO_2 pressure [Elsden, 1938]. We regard this as evidence in favour of the view that reaction 1 is a stage in the succinate formation not only from pyruvate but also from hexoses. This hypothesis postulates that the succinate formation from pyruvate, oxaloacetate, malate and fumarate should be at least as rapid as the succinate formation from the other succinate-forming substrates. Data in the papers of Elsden [1938] and Krebs [1937] fulfil this postulate.

Whilst it is by no means impossible that there is a second mechanism of succinate synthesis from carbohydrate in animal tissues or in *B. coli*, there is nothing, as far as we can see, to be said in favour of such an alternative. The facts can all be explained by the mechanism proposed in this paper.

Summary

1. Suspensions of minced pigeon liver form the same substances from added pyruvate and from added oxaloacetate, viz. α-ketoglutarate, fumarate, malate, succinate and citrate. Various explanations are discussed. The only satisfactory explanation is the assumption that a formation of oxaloacetate from pyruvate and CO_2 (reaction 1) takes place.

2. The rate of pyruvate usage in pigeon liver depends on the concentrations of CO_2 and bicarbonate. This supports the view that CO_2 takes part in pyruvate removal as an active metabolite, as indicated by reaction 1.

3. In vitamin B_1-deficient pigeons the rate of pyruvate usage of liver suspension is greatly reduced. The normal rate is restored by the addition of pure vitamin B_1. Under the same conditions pyruvate usage in muscle suspensions, i.e. pyruvate usage through the citric acid cycle, is not increased by the vitamin. It follows that the vitamin takes part in a reaction present in liver, but absent from muscle, and by the method of elimination the conclusion is reached that the vitamin catalyses reaction 1, the "carboxylation" of pyruvate. It is not excluded that in addition the vitamin takes part in the synthesis of acetoacetate from pyruvate (see § I, 6).

4. In *B. coli* fumarate and malate are formed (together with succinate) when pyruvate is anaerobically fermented. This supports the view, previously expressed by Wood & Werkman, that reaction 1 occurs in bacteria. The CO_2 fixation and the equivalence between CO_2 fixation and succinate formation in

propionic acid bacteria (Wood & Werkman) and the effect of CO_2 on the succinate formation in *B. coli* (Elsden) are satisfactorily explained by this assumption.

This work was aided by grants from the Rockefeller Foundation and the Medical Research Council.

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Note added on 31 October 1940. With the help of radio-active carbon (C_{11}) Ruben & Kamen (*Proc. Nat. Acad. Sciences*, **26**, 418, 1940) have recently succeeded in demonstrating the "assimilation" of CO_2 by rat liver and other heterotrophic cells. Attempts to identify the organic molecules containing the radio-active carbon atoms were unsuccessful. It is to be expected that active carbon will be found in the organic acids formed in the course of the citric acid cycle.

165. THE PHYSIOLOGICAL ACTION OF ABNORMALLY HIGH TEMPERATURES ON POIKILOTHERMIC ANIMALS

3. SOME CHANGES OCCURRING IN THE PHOSPHORUS DISTRIBUTION OF THE HAEMOLYMPH OF INSECTS UNDER THE INFLUENCE OF ABNORMALLY HIGH TEMPERATURE

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(*Received 24 September 1940*)

In the first paper of this series [Fraenkel & Hopf, 1940] it has been shown that the degree of saturation of the phosphatides of fully grown larvae of *Calliphora erythrocephala* Meig. and *Phormia terra-novae* R.D. is dependent on the temperature at which these larvae have been bred, irrespective of species. This means that these blowfly larvae will give the same iodine value for the fatty acids of their phosphatides for the same temperature of breeding, no matter to which of the two closely allied species they belong. The heat adaptation of these larvae was also tested and it was found that the larvae bred at higher temperatures were more resistant to heat treatment than larvae bred at lower temperatures. These results were discussed in the light of the lipoid liberation theory formulated by Heilbrunn [1924] and by Belehradek [1931] which attributes the cause of death from exposure to abnormally high temperatures to a "liberation" of the lipoids and explains the heat adaptation by the raising of the melting point in the more saturated fats of the larvae bred at high temperatures. It was pointed out by Fraenkel & Hopf [1940] that, though this theory seemed to be supported by the correlation between the temperature of breeding and the degree of unsaturation in the animals in question, it left unexplained the phenomenon that the two species, while showing the same degree of unsaturation at the same temperature of breeding, had a vastly different resistance to high temperatures. Heat resistance therefore cannot depend solely on the nature of the lipoids concerned and investigations extending over a wider field were clearly desirable. The present communication records the results of the changes occurring in the haemolymph (body fluid) under the influence of abnormally high temperatures.

The reasons for choosing the haemolymph as material for analysis are as follows. The haemolymph is the only body fluid in insects. If metabolic processes are involved in heat injury it is probable that some evidence of the changes will be found in the blood. Furthermore, the haemolymph is obtained with comparative ease from the body and is present in large amounts in the larvae of *Calliphora erythrocephala* and *Phormia terra-novae*, so that its analysis can be conveniently carried out. The need for a survey of the fate of the P distribution during heat injury was shown by the observation (to be described

below) that the total P content of the blood seemed to depend on the temperature at which the larvae were kept previous to the extraction of the haemolymph.

Heat treatment of the larvae and preparation of the haemolymph for analysis

Before obtaining the blood for analysis it was important that the insects should be in a reproducible condition of heat injury and that strictly standard conditions should be observed throughout. It was not thought advisable to use the larvae in a state in which they had actually been killed by heat, as post-mortem changes were then likely to have occurred which had no direct connexion with the heat treatment given. It was therefore found best to use the larvae while they were still alive, though often in a state of heat rigor in which the high temperature had already produced irreversible injuries, so that normal flies would not develop from larvae thus treated.

Before death takes place in heat-treated larvae they develop heat rigor, a state in which they are motionless and fully extended, but will still react ("twitch") when some outside stimulus, such as pressing between the fingers, is applied. Unfortunately this state is not equally reversible in the two species, for while normal flies will seldom develop from *Calliphora* larvae after heat rigor, recovery is general in those of *Phormia* if the rigor has not lasted too long. Table 1 shows the differences between the two species. In these experiments

Table 1. *Tests of survival from heat rigor*

	Phormia at 45°				*Calliphora* at 40°			
	½ hr.		¾ hr.		½ hr.		¾ hr.	
	A	B	A	B	A	B	A	B
"Twitching"	50	48	22	15	44	38	0	0
Normal pupae	42	42	4	0	3	5	0	0
Flies	38	40	4	0	1	0	0	0

a temperature was chosen which was known to kill insects exposed to it within 5 hr., namely 45° for *Phormia* and 39° for *Calliphora* [Fraenkel & Hopf, 1940]. In each case two batches of 50 larvae were used: one was restored to normal condition when 25 of the larvae had been motionless for 15 min. and the other after 45 min. further exposure to heat. A record was then taken of the number of larvae showing "twitching" 1 hr. after return to normal conditions, of the number of normal pupae formed and of the number of flies emerging from them. The experiments were carried out in duplicate, a different brood being used each time. The larvae were reared at 27° and used fully grown.

It was decided that in the following experiments the *Phormia* larvae should be subjected to heat treatment until they passed into full heat rigor, while those of *Calliphora* should be given treatment sufficient only to produce the first evidences of this state. Both the temperature and time factors were accurately controlled.

The method of heat treatment has been fully described in the previous paper. After treatment the larvae were removed from the bottles, washed with distilled water, dried by gently rolling them between two sheets of filter paper and the body fluid was extracted in the following way.

A mixture of 9 parts of paraffin (Shell 24210) and 1 part of toluene was rendered air-free by allowing it to stand *in vacuo* for several hours until no more bubbles appeared. This fluid was neutral and was used to keep the blood ex-

tracted from the larvae free from contact with air, dust or bacteria. A 10 ml. conical centrifuge tube was filled to the brim with this mixture and the tube placed upright in a conical flask, which served as a receptacle for the overflow when the blood was added.

Each larva in turn was taken up separately by the hind end and brought near the surface of the liquid. By pressing the hind end between the index finger and the thumb of the left hand all the blood collected in the front end of the animal. On puncturing the front end with a small sterilized scalpel or needle, a drop of blood issued from the wound, which was brought into contact with the surface of the paraffin-toluene mixture, whereupon the drop sank to the bottom of the tube. The blood slowly accumulated there, causing an overflow of the protecting liquid. As it was essential to have the haemolymph uncontaminated by the gut of the larva, the incision was made at the mesothoracic segment in a slightly tangential direction.

In order to obtain 1 ml. of haemolymph, 90–110 larvae had to be treated in this way, heat-treated insects giving slightly less blood than the untreated controls.

After sufficient material had been collected the tubes were spun on a centrifuge for a few minutes. The blood corpuscles then formed a deposit and a layer of fat globules and fragments of body fat was found floating on top of the haemolymph proper. The latter appeared as an almost clear pale yellow fluid, usually somewhat darker when derived from heat-treated larvae than from the controls. The relative amounts of the three above-mentioned layers was approximately: serum 96 %, corpuscles 3 % and fat 1 % in the untreated larvae, while the fat content in the "sub-killed", i.e. irreversibly injured but still living, larvae was often as much as 2 %. After the larvae were dead the fat content of the haemolymph rapidly increased, fat being one of the first tissues of the insect body to disintegrate.

A graduated pipette was then carefully inserted into the tube and the fat layer was gently pushed aside so that the serum could be drawn off and analysed without coming into contact with air. On standing in the centrifuge tube for several hours the uppermost layers of the blood, where it came into contact with the paraffin, slowly blackened. Whether this blackening, which is doubtless a tyrosinase reaction, is caused by oxygen from the air diffusing through the paraffin or by an inter-surface action from the junction of the haemolymph with the paraffin-toluene mixture has not been determined. The blood serum thus obtained was in one instance used for a determination of the specific gravity and dry weight, the results being given in Table 2.

Table 2. *Specific gravity and dry weight of blood serum*

		45° for 4 hr.	Control
Phormia	Sp. gr.	1·029	1·018
	Dry wt.	17·25 %	16·55 %
		40° for 4 hr.	Control
Calliphora	Sp. gr.	1·025	1·021
	Dry wt.	17·10 %	16·23 %

Phosphorus distribution in the haemolymph of heat-treated and of normal larvae

If the total P in the blood of heat-treated larvae is compared with the value for unheated controls, it is found that a considerable increase has taken place during the exposure to high temperatures (see Table 3). During the present

work a batch of untreated larvae was in one instance kept in a refrigerator for 2 days as it could not be worked up at once and it was desired to arrest larval development and prevent pupation. The blood P of this particular batch was even lower than that of larvae treated at the normal temperature. The experiment was then repeated with aliquots of larvae from one brood, one sample being heat-treated, one exposed to cold and the last kept at 27°—the temperature of breeding—and it was found that these conditions produced different total P contents in the haemolymph. That the processes involved were really connected with life functions was proved by the fact that the value for heat-killed larvae was the same before and after being kept in the refrigerator for a period of 2 days.

Table 3. *The dependence of the total P of blood on external temperature*

		Batches of larvae from the same brood mg. P/ml.	
		A	B
Phormia	Heat rigor at 45° (2½ hr.)	1·08	0·93
	Normal	0·74	0·69
	Kept at 4° for 2 days	0·68	0·62
Calliphora	Heat rigor at 41° (2½ hr.)	1·22	1·30
	Normal	0·91	1·04
	Kept at 4° for 2 days	0·85	0·92

In view of the fact that P plays an important part in such vital functions as carbohydrate metabolism and coenzyme activities a survey of the fate of various P compounds during heat treatment was undertaken. A combination of the methods applied by Needham & Needham [1930] and by Needham *et al.* [1937] to embryological work was used in the following analyses, P being estimated throughout by the method of Martland & Robison [1926].

The lipoid P (phosphatides) was determined by extraction of the haemolymph with alcohol and ether. For this purpose a measured quantity of blood, usually 2 ml., was mixed with analytical P-free $CaSO_4$ and allowed to dry over H_2SO_4 for 2–3 weeks. From the loss in weight during this operation the total blood solids were calculated. The dry mixture was then transferred to a Soxhlet thimble and extracted for 4 days with 97 % alcohol, followed by ether (sp. gr. 0·720) for an equal time. The alcoholic extract was taken to dryness and the residue extracted 6 times with boiling ether. The P in the combined ether extracts was regarded as the total lipoid P.

The residue in the Soxhlet thimble was then extracted 5 times in the cold for periods of ½ hr. each with 1 % HCl and 5 times for similar periods with distilled water. Needham & Needham [1930] combined this acid-soluble extract with the ether-insoluble residue from the alcohol extract, thus obtaining a total acid-soluble fraction in which they estimated the inorganic P, the pyrophosphate-P and the stable organic P. Nevertheless, for the purposes of the present work it was found more expedient to use the method described by Needham *et al.* [1937], involving the use of trichloroacetic acid, for the acid-soluble fraction, since this gives a more detailed and physiologically more interesting partition of the P. Experience showed that the two methods give values for the total acid-soluble P which agree within a limit of error of 6 %. The $CaSO_4$ in the thimble now retained only the protein compounds of P, and phosphoprotein-P and nucleoprotein-P were estimated in the usual way.

The acid-soluble P was fractionated as follows. 5 ml. of haemolymph from the same tube as that from which the material for the lipoid extraction was taken were precipitated with 25 ml. of ice-cold 5% trichloroacetic acid in a tube standing in a beaker with ice. The tube was allowed to stand in the refrigerator for 6 hr., after which the contents were filtered through an asbestos mat under pressure and the precipitate washed several times with small amounts of ice-cold trichloroacetic acid and water. The precipitate was rejected and the filtrate made up to 50 ml. The total acid-soluble P was estimated in aliquots of 1 ml., each ml. at this stage representing 0·1 ml. of haemolymph.

The remaining solution was then transferred to a centrifuge tube and fractionated according to the procedure of Needham *et al.* [1937], modified in some minor points to suit the material under investigation. The barium precipitate gave values for inorganic P, adenylpyrophosphate-P, hexosediphosphate-P and non-hydrolysing P, while the barium filtrate gave values for phosphagen-P and hexosemonophosphate-P; the last value in each group probably represented, in part, esters other than those of hexose.

Table 4. *P distribution in the haemolymph of heat-treated* Calliphora *larvae*

	15 hr. at 38·5°			1 hr. at 42°			Control		
	A	B	C	A	B	C	A	B	C
Total P	1·35	1·20	1·47	1·19	1·02	1·08	1·02	0·92	0·98
Lipoid P	0·16	0·16	0·14	0·12	0·14	0·12	0·10	0·09	0·10
Total acid-soluble P	1·10	0·98	1·25	0·95	0·82	0·83	0·75	0·71	0·76
Inorganic P	0·76	0·70	0·82	0·60	0·52	0·48	0·45	0·42	0·38
Adenylpyrophosphate-P	0·14	0·12	0·19	0·14	0·15	0·17	0·10	0·08	0·14
"Hexosediphosphate-P"	0·04	0·03	0·07	0·04	0·02	0·06	0·04	0·05	0·07
Non-hydrolysing P	0·05	0·04	0·08	0·05	0·03	0·08	0·05	0·06	0·07
Phosphagen-P	0·02	0·02	0·02	0·04	0·02	0·04	0·04	0·05	0·09
Hexosemonophosphate-P	0·05	0·04	0·07	0·05	0·04	0·02	0·05	0·06	0·01
Phosphoprotein-P	—	Trace	—	Trace	Trace	Trace	—	—	—
Nucleoprotein-P	Trace	Trace	—	Trace	Trace	—	—	Trace	—

Table 5. *P distribution in the haemolymph of heat-treated* Phormia *larvae; mg. P./ml.*

	15 hr. at 42·5°			1 hr. at 46°			Control		
	A	B	C	A	B	C	A	B	C
Total P	0·85	0·93	1·04	0·62	0·85	0·90	0·52	0·68	0·72
Lipoid P	0·08	0·11	0·13	0·04	0·08	0·09	0·04	0·06	0·08
Total acid-soluble P	0·67	0·71	0·82	0·50	0·69	0·73	0·41	0·54	0·58
Inorganic P	0·42	0·48	0·52	0·27	0·35	0·41	0·22	0·25	0·30
Adenylpyrophosphate-P	0·07	0·09	0·10	0·07	0·09	0·09	0·04	0·06	0·08
"Hexosediphosphate-P"	0·04	0·03	0·05	0·03	0·05	0·03	0·04	0·04	0·03
Non-hydrolysing P	0·04	0·04	0·05	0·03	0·05	0·06	0·04	0·05	0·03
Phosphagen-P	0·02	0·01	0·03	0·04	0·04	0·07	0·04	0·05	0·07
Hexosemonophosphate-P	0·03	—	0·05	0·01	0·02	0·04	0·01	Trace	0·03
Phosphoprotein-P	—	—	—	—	Trace	—	—	—	—
Nucleoprotein-P	Trace	Trace	Trace	—	Trace	Trace	—	—	—

In Tables 4 and 5 three sets of analyses are given for both *Phormia* and *Calliphora* larvae. They do not represent all the experiments carried out, but they constitute the only complete sets of results available and all the others were in accordance with those reproduced. In each case a brood of larvae was divided into 3 batches: one was heated to the "subkilled" state for 1 hr., the second to a temperature suitable to produce the same state within 15 hr., and the third kept as a control at the temperature of breeding (27°). Experiments have been carried out on larvae bred at higher and lower temperatures, and

although the chemical analyses are not yet completed the results so far obtained do not show any divergence from those given with larvae bred at 27° as long as the temperature employed produced the same state of heat injury in the same time of treatment as that used in the tabulated experiments.

In spite of all precautions to ensure the same state of heat injury in all cases, it was generally found that larvae treated for a long time at the lower temperature would survive the heat treatment for several hours longer than those treated at the high temperature. This may be attributed to the fact that at the high temperature the time increment between complete recovery and complete death is much smaller than at low temperatures, and that therefore the heat treatment was administered slightly on the excessive side to make sure that most of the insects were, in fact, effectively heat-injured.

In Tables 4 and 5 all numbers denote mg. per 1 ml. of haemolymph and are calculated to the nearest 0·01 mg.; A, B, C, refer to different broods of larvae. The results show for both species an increase in lipoid P, inorganic P and adenylpyrophosphate-P after heat treatment. It will also be noticed that, with the exception of the adenylpyrophosphate-P, the increase is more pronounced in larvae treated at the lower temperature for the longer time.

Discussion

The above-mentioned results suggest that the P distribution in the haemolymph is dependent on the temperature to which the insect has been exposed before the blood is collected rather than on the temperature of breeding. The increase in P content after heat treatment of the larvae is not due merely to the increased concentration of the blood, for the total P increased by 15–30 % while the dry weight increased by only about 3 %.

It will be noted that there is a considerable increase in lipoid P, representing on the average about a third of the total increase. This might suggest that fat has been melted or otherwise liberated from the body cells, thus providing a simple explanation of the heat injury. Against this view stands the fact that the increase in lipoid P is greater in larvae exposed for a long time to a low lethal temperature than in others treated for a shorter time at a higher temperature, though the heat treatment was, if anything, slightly more effective in the latter case.

A striking rise in inorganic P is evident, and the assumption that this is connected with the buffering systems of the haemolymph will be discussed in a later communication.

The method of fractional hydrolysis used to separate the esters precipitable with barium acetate is probably not ideal and there remains the possibility that other compounds besides those mentioned in the tables have been hydrolysed under the conditions employed. The figures quoted therefore have only a comparative value.

The adenylpyrophosphate-P has also risen quite considerably during heat injury and as adenylpyrophosphate is known to be a constituent of several enzyme systems it may be that heat injury produces a certain amount of enzyme activation in the haemolymph, a supposition which will be followed up in future work.

As has already been pointed out, the value recorded as hexosediphosphate-P represents only half of the total phosphate of this compound, for after 100 min. hydrolysis only one of the two phosphate groups attached to the carbohydrate has been split off, leaving the more resistant hexosemonophosphate to be

estimated as non-hydrolysing P. This should, of course, not be confused with the hexosemonophosphate originally present, which is not precipitated by barium acetate. No significant change appears to occur in the hexosediphosphate-P during heat injury. The amount of non-hydrolysing P is slightly larger in all cases than that of the hexosediphosphate-P, which suggests the presence of other P compounds as well as hexosediphosphate. As regards the group of P compounds not precipitable with barium acetate, it is interesting that phosphagen is the only fraction which actually decreases during heat treatment. However, phosphagens are known to be very unstable and to hydrolyse easily, so that it is very probable that heat injury produces an actual breakdown of phosphagen in the blood. The nature of the phosphagen present in the larvae has not been investigated, but there is no evidence for an occurrence of creatine phosphates in animals of related phyla, while Baldwin & Needham [1933] have shown that the phosphagen in the muscle of flies is arginine phosphate.

It is impossible to say whether the slight increase noticed in the hexosemonophosphate fraction in some of the cases examined represents a metabolic effect of heat treatment, especially as the small values impair the reliability of the results. In the author's opinion this increase, where it does occur, is more likely to be concerned with the incidental carbohydrate metabolism of the insects than with adaptation to high temperature.

Comparing the two species it appears that the same tendency is observable in each case. The actual recorded changes for *Calliphora* are higher than those for *Phormia* but the relative increase is more pronounced in the latter. Thus the average increase in lipoid P after long treatment is from about 0·1 to 0·15 mg. for *Calliphora*, and from 0·06 to 0·11 mg. for *Phormia*, while the corresponding increases for acid-soluble P are approximately from 0·75 to 1·10 mg. and from 0·5 to 0·75 mg. respectively.

Treatment for a short time at a high temperature produces less pronounced results than treatment for a long time at a somewhat lower temperature except in the case of the adenylpyrophosphate-P, which increased equally in both cases. This suggests that, if the larger increase at lower temperatures is to be attributed chiefly to the longer time of exposure, the adenylpyrophosphate (coenzymic) groups are probably liberated first from the cells, and that this process ceases on prolonged treatment.

Regarding the cause of the P increase as a whole, there appear to be two possibilities:

(*a*) the increase is due merely to a higher solubility at higher temperatures, accentuated by the greater permeability which heat-affected cells may possibly show, or

(*b*) this increase is connected with a series of metabolic processes instigated by the reaction of the organism to high temperatures, and therefore constituting an adaptation of the organism to the change in environmental conditions.

While the first possibility seems to explain satisfactorily the increase in inorganic P well enough, and might also perhaps explain the increase in coenzyme-P, the increase in phosphatide content is in strict contradiction to it. The total fat content of the haemolymph does not increase nearly as much as would account for the increase in phosphatide, were this product liberated in proportion to the other lipoids that occur in the body. We must therefore assume that there is a preferential release of phosphatide as compared with other fats. Furthermore, the phosphatides are known to form very stable complexes with proteins in the protoplasm, so that we may conclude that this particular increase is due to metabolic processes and not to a physically

indiscriminate dissolving of tissue material. Similarly the apparently equal behaviours of inorganic and lipoid P compounds make it possible that a connexion exists between the two. We may therefore assume that the changes described above do, in fact, represent an adaptation of the organism towards a change in its environmental conditions.

SUMMARY

Exposure of the larvae of *Calliphora erythrocephala* and *Phormia terra-novae* to abnormally high temperatures produces an increase in the lipoid P, the inorganic P and the adenylpyrophosphate-P of the haemolymph. The function of the two latter groups might be regarded as being connected with buffering and coenzymic activities respectively. The increase is more pronounced for a long exposure to low lethal temperatures than for a short exposure to high lethal temperatures, except in the case of the adenylpyrophosphate-P which increases equally in both cases and is assumed to be liberated at an early stage.

The method of obtaining the blood and of standardizing the heat treatment is described.

The author wishes to express his sincere gratitude to Prof. J. W. Munro, who originally suggested this work and made it possible for him to undertake it; to Prof. A. C. Chibnall for many valuable suggestions and for the hospitality of the Biochemistry Department; to Dr G. Fraenkel, under whose general supervision this work was carried out and to whose work on heat adaptation the present paper constitutes a sequel; and to Dr J. Needham and Dr H. W. Buston for much kindness and valuable advice. The costs of this investigation was partly defrayed by a grant from the Agricultural Research Council

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166. A CHEMICAL STUDY OF THE BEARING OF DECAY BY *PHELLINUS CRYPTARUM* KARST. AND OTHER FUNGI ON THE DESTRUCTION OF WOOD BY THE DEATH-WATCH BEETLE (*XESTOBIUM RUFOVILLOSUM* DE G.)

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(*Received 30 August 1940*)

The chemical aspect of the destruction of oak wood by the death-watch beetle was first discussed some years ago by one of the authors [Campbell, 1929] and the conclusion was then reached that the larval diet consisted essentially of the carbohydrates, cellulose and hemicelluloses. Since, however, the composition of the original wood and the loss in weight due to insect attack could not be determined from the material available, it was pointed out that the full effectiveness of the application of chemical analysis to the elucidation of feeding habits could not be realized because the true extent of depletion in each wood component could not be assessed. The general conclusions of this preliminary work were confirmed in the following year by Ripper [1930] and more recently by Norman [1936]. In all of this early chemical work little or no significance was attached to the long established observation that the natural habitat of *Xestobium* is in dead—and in many instances decayed—parts of hardwood trees. Fisher [1935] came to the important conclusion that fungal decay, among other factors in a suitable timber, can have a marked influence on the duration of the life-cycle of the insect, and in later work [Fisher, 1937; 1940] was able to rear it under laboratory conditions in oak sapwood which had previously been decayed by various fungi in pure culture. Fisher established that as the severity of fungal decay increased, the duration of the life cycle of the insect became less. The first indication from the purely chemical standpoint that, in wood attacked by the death-watch beetle, there can be fungal decomposition of the same order both in the material consumed by the larvae and in the residual wood which is not comminuted by them, is afforded by Norman's results [1936]; but, as will be shown later, this author apparently failed to appreciate their full significance. Thanks to the aforementioned work by Fisher it is now possible to rear *Xestobium* in large numbers and in reasonably short periods of time in weighed samples of wood of known composition, so that the essential prerequisites originally postulated [Campbell, 1929] for the effective chemical investigation of feeding habits have now been met.

The white rot fungus, *Phellinus cryptarum*, has from time to time been identified in wood infested with *Xestobium*, and in the first part of the present study the ultimate chemical effect of this fungus on wood has been determined for the first time. The bearing of fungal decay on the availability of oak wood to *Xestobium* has been examined from a number of points of view and the

results of previous chemical work on the feeding habits of the insect have been carefully reconsidered.

The experimental material was made available through the co-operation of Mr K. St G. Cartwright of the Mycology Section and Dr R. C. Fisher of the Entomology Section of this Laboratory, to whom grateful acknowledgement is here made. The original wood consisted of a series of carefully matched pieces of air-dried English oak (*Quercus* sp.) sapwood, each of which measured approximately $15 \times 4 \times 4$ cm. and weighed approximately 120 g.

Experimental

It has been observed previously [Campbell, 1929] that the frass of *Xestobium* contains a considerable proportion of material which passes a standard 120-mesh screen. It was therefore necessary to ensure, by mechanical grinding where necessary, that all the material submitted to analysis passed this screen despite the conclusion of Campbell & Bryant [1937] that grinding of wood to a fine powder for most analytical purposes is open to certain objections. These objections are entirely overcome, however, if the analytical data which follow are only used for comparative purposes among themselves.

Four pieces of the original wood were selected at random and comminuted to pass the 120-mesh screen. The analysis of the thoroughly mixed flour is given in Table 1. In this table are also recorded the analyses of a series of samples

Table 1. *Composition of oak sapwood at different stages of decay by* Phellinus cryptarum

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Loss of wt. by decay (% original sound wood)	(Sound wood)	14·9	22·65	30·8	36·9	44·8	51·2	60·3	74·6
Soluble in:									
Cold water	4·00	6·78	6·01	7·17	6·97	6·76	7·03	8·18	10·59
Hot water	6·45	10·53	8·45	9·65	9·19	9·11	10·72	12·21	16·54
1% NaOH	24·18	30·02	27·45	30·20	29·37	31·07	31·62	35·20	40·20
Alcohol-benzene	2·54	4·04	3·23	3·80	4·27	3·43	4·86	5·53	5·71
Cellulose	51·30	51·60	52·65	54·05	53·68	55·21	50·40	50·96	51·65
Pentosans:									
In cellulose	13·26	13·93	13·42	12·90	14·30	13·50	12·24	13·02	11·50
Not in cellulose	11·31	11·32	12·07	11·37	11·26	10·87	12·27	11·35	9·92
Total	24·97	25·24	25·49	24·28	28·55	24·36	24·51	24·37	21·42
Lignin	19·80	18·65	18·57	16·78	18·22	16·99	18·63	17·88	14·85
Methoxyl:									
In lignin	4·20	3·88	3·93	3·53	3·76	3·57	3·77	3·69	3·01
Not in lignin	2·16	2·21	1·37	1·98	2·05	1·78	2·23	2·51	1·99
Total	6·36	6·09	5·30	5·51	5·80	5·34	6·01	6·20	5·00

which had been exposed to decay by the fungus. A further series of pieces of the original wood which had been exposed to fungal decay and then sterilized by dry heat at 105° were selected in groups of 4 for exposure to insect attack according to the percentage loss in weight sustained during decay. The technical operations involved in the biological aspects of this work will be described elsewhere. By plotting the percentages of the various wood components against the losses in weight (Table 2) a series of curves was obtained from which the average composition on a sound wood basis of any set of decayed samples submitted to insect attack could be determined. In all, seven groups of specimens

Table 2. *Composition of oak sapwood at different stages of decay by* Phellinus cryptarum

Results expressed as percentages by weight of oven-dry original sound wood

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Loss by decay	(Sound wood)	14·9	22·65	30·8	36·9	44·8	51·2	60·3	74·6
Soluble in:									
Cold water	4·00	5·77	4·65	4·96	4·40	3·73	3·43	3·26	2·69
Hot water	6·45	8·96	6·54	6·68	5·80	5·03	5·23	4·85	4·20
1% NaOH	24·18	25·55	21·23	20·90	18·54	17·15	15·43	13·97	10·21
Alcohol-benzene	2·54	3·44	2·50	2·63	2·69	1·89	2·37	2·20	1·45
Cellulose	51·30	43·91	40·73	37·41	33·87	30·47	24·59	20·23	13·12
Pentosans:									
In cellulose	13·26	11·85	10·38	8·93	9·02	7·45	5·97	5·17	2·92
Not in cellulose	11·31	9·63	9·34	7·87	7·10	6·00	5·99	4·50	2·52
Total	24·97	21·48	19·72	16·80	16·12	13·45	11·96	9·67	5·44
Lignin	19·80	15·87	14·36	11·61	11·47	9·38	9·09	7·09	3·77
Methoxyl:									
In lignin	4·20	3·30	3·04	2·44	2·37	1·97	1·84	1·47	0·76
Not in lignin	2·16	1·88	1·06	1·37	1·29	0·98	1·09	0·99	0·51
Total	6·36	5·18	4·10	3·81	3·66	2·95	2·93	2·46	1·27

which had been exposed to insect attack after having been decayed by *Phellinus cryptarum* were examined. The loss in weight due to fungal decay being known, the net loss due to insect attack was determined for each group. In each case frass was separated mechanically from the decayed wood which had not been disintegrated by the larvae, in order to obtain the data recorded in Table 3.

Table 3. *The effect in terms of loss in weight of* Xestobium rufovillosum *on oak sapwood at various stages of decay caused by* Phellinus cryptarum

Average loss of weight by decay (% sound wood)	25·86	30·21	36·48	40·04	50·47	59·76	73·40
Decayed wood exposed to insect attack (g., oven-dry)	323·23	266·27	251·56	245·50	150·63	126·71	90·94
Undisintegrated wood after insect attack (g., oven-dry)	229·67	164·4	136·40	134·40	63·61	56·63	31·41
Wood disintegrated (by difference) (g., oven-dry)	93·56	101·87	115·16	111·10	87·02	70·08	59·53
Frass (g., oven-dry)	60·53	72·63	87·60	87·60	71·62	58·39	51·44
Loss by insect attack (by difference) (g., oven-dry)	33·03	29·24	27·56	23·50	15·40	11·69	8·09
Loss by insect attack as % wood disintegrated (abstraction coefficient)	35·30	28·70	23·93	21·15	17·70	16·68	13·59

In each of two cases proportional parts of frass and undisintegrated wood were comminuted, mixed and analysed (Table 4) and a sample of frass from each of the two groups in question was analysed separately (Table 5) in order to obtain somewhat magnified values for the small percentage losses incurred during the insect attack.

For comparative purposes the analytical processes applied to the wood samples were also applied to a sample of *P. cryptarum* mycelium taken from the surfaces of some of the oak sapwood specimens. The mycelium was difficult to comminute and no attempt was made to grind it to pass a 120-mesh screen. It

Table 4. *Composition of oak sapwood decayed by* Phellinus cryptarum, *before and after attack by* Xestobium rufovillosum

Results expressed as percentages by weight of oven-dry original sound wood. B = before insect attack. A = after insect attack. Figures in column B obtained graphically from the results in Table 2.

Loss by decay	(1) 36·48		(2) 40·04	
	B	A	B	A
Loss by insect attack	—	6·07	—	5·08
Soluble in:				
Cold water	4·44	3·84	4·26	3·56
Hot water	6·00	4·94	5·80	4·56
1% NaOH	18·84	16·90	18·05	16·54
Alcohol-benzene	2·52	1·93	2·41	1·58
Cellulose	34·28	30·84	32·65	30·79
Pentosans:				
In cellulose	8·51	7·96	7·99	7·42
Not in cellulose	7·21	6·68	6·80	5·57
Total	15·72	14·64	14·79	12·99
Lignin	11·19	10·44	10·33	9·52
Methoxyl:				
In lignin	2·36	2·17	2·18	2·06
Not in lignin	1·30	1·17	1·23	0·91
Total	3·66	3·34	3·41	2·97

Table 5. *Analytical comparison of decayed wood available to* Xestobium, *the material digested therefrom, and the mycelium of* Phellinus cryptarum

All % are based on oven-dry material. Loss of weight by fungal decay: (*a*) 36·48% of sound wood; (*b*) 40·04% of sound wood.

	Decayed wood ingested by insect Calc. from graphs, %, own basis (1)		Frass % of decayed wood ingested (2)		Material digested by insect: % of decayed wood ingested (by difference) (3)		Material digested by insect: %, own basis (4)		Mycelium %, own basis (5)
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	
Soluble in:									
Cold water	6·99	7·11	5·42	5·14	1·57	1·97	6·56	9·32	6·71
Hot water	9·45	9·67	7·16	6·85	2·29	2·82	9·57	13·33	11·32
1% NaOH	29·66	30·11	23·76	24·80	5·90	5·31	24·60	25·11	50·29
Alcohol-benzene	3·97	4·02	2·69	2·04	1·28	1·98	5·35	9·36	6·50
Cellulose	53·96	54·46	40·87	44·54	13·09	9·92	54·68	46·90	61·54
Pentosans:									
In cellulose	13·40	13·32	10·91	10·35	2·49	2·97	10·40	14·05	2·36
Not in cellulose	11·35	11·35	7·37	7·87	3·98	3·48	16·63	16·45	3·75
Total	24·75	24·67	18·28	18·22	6·47	6·45	27·04	30·50	6·11
Lignin	17·61	17·23	14·81	13·59	2·80	3·64	11·70	17·21	3·30
Methoxyl:									
In lignin	3·72	3·64	3·13	2·90	0·59	0·74	2·47	3·50	0·05
Not in lignin	2·05	2·05	1·40	1·28	0·65	0·77	2·72	3·64	0·52
Total	5·76	5·69	4·53	4·18	1·23	1·51	5·14	7·14	0·57
Methoxyl in lignin, % lignin	21·1	21·1	21·1	21·3	—	—	21·1	20·3	1·6

is not claimed either that the data obtained from mycelium represent its exact composition or that the substances estimated are identical with those present in wood.

Nitrogen determinations (Table 6) were carried out by the method recommended by Campbell & Hanna [1937] on mycelium, sound wood, a sample of decayed wood and a sample of decayed wood after insect attack. The mycelium

Table 6. *Total nitrogen contents of oak sapwood,* Phellinus cryptarum *mycelium and* Xestobium rufovillosum *larvae respectively*

Results for wood expressed as percentages by weight of original sound wood

	N, % oven-dry weight
Sound wood	0·16
Wood decayed to a loss in weight of 36·48 % before insect attack	0·12
Wood decayed to a loss in weight of 36·48 % after insect attack	0·10
Mycelium of *P. cryptarum*	0·68
Xestobium larvae	3·91

was found to contain 0·5 % of its oven-dry weight of acetyl groups, the method described by Schorger [1926, p. 530] for wood being used for the determination.

Discussion

As a result of previous studies on the chemistry of the white rots of wood it has been shown [Campbell, 1930; 1931; 1932] that, on chemical grounds, they can be subdivided into three classes. The present results (Tables 1 and 2) show that the white rot produced by *Phellinus cryptarum* falls into the class in which cellulose and lignin are decomposed simultaneously from the commencement of decay and furthermore that the various wood components are decomposed in approximately the same proportions in which they occur in the original wood. For this reason, the existence and extent of decay are not readily revealed by chemical analysis of the decayed wood alone. The highest loss in weight due to decay observed in the present investigation was approximately 75 %, and since the decayed wood at this stage still contained lignin, simple but conclusive proof is here afforded that, in white rots of wood, lignin cannot constitute the sole source of fungal nourishment.

Turning now to the material assimilated from the decayed wood by the insect, it is observed from Table 4 that the larvae digest a proportion of each of the components determined by analysis. The composition of the diet may also be assessed by comparing the composition of the frass, as distinct from that of the total residue after insect attack, with the composition of the decayed wood submitted to attack (Table 5).

Prior to the present investigation the opinion has been expressed [Mansour & Mansour-Bek, 1934, and others] that lignin, as such, cannot be assimilated by insects, although Norman [1936] apparently accepts this view with some reluctance in the case of *Xestobium*. Certain authors have expressed the composition of the frass of wood-boring insects on the basis of unattacked wood by assuming that the ash constituents [Falck, 1930], or even the lignin itself, have remained entirely unaffected. The assimilation of lignin in addition to extractives and the carbohydrates of wood by *Xestobium* is one of the most important facts

brought to light in the present study. It is probable that the ability of *Xestobium* to break down lignin does not depend on the previous action of any particular type of fungus. Since, moreover, the percentage of methoxyl groups in the lignin removed by the larvae (Table 5) is approximately the same as the corresponding value for the lignin of decayed wood, it is deduced that decomposition is not confined to any particular portion of the lignin molecule. The view that a possible carbohydrate or other non-aromatic portion of the molecule is preferentially digested cannot therefore be sustained.

It cannot be overlooked that lignin may be decomposed in the larval gut and its products not wholly assimilated so that material derived therefrom may occur in the frass and yet not be determined on analysis. That such a condition does not obtain, however, is proved by further consideration of the data in Table 5. The sums of the losses due to insect attack of hot-water extractives, cellulose, pentosans not in cellulose, lignin, and methoxyl not in lignin as calculated by difference from columns (1) and (2) amount to 20·63 and 22·81 % of the respective samples of decayed wood submitted to insect attack, and these agree closely with the respective percentage losses in weight (21·15 and 23·95) which were determined directly. Had the frass contained unassimilated products of lignin, the above calculated percentage losses due to insect attack would have been appreciably greater instead of less than the determined losses.

The question of the food value to the insect of the fungal mycelium in the decayed wood has not been overlooked. In view of the apparent composition of the mycelium of *Phellinus cryptarum* (Table 5) it is obviously impossible to make a direct determination of the amount present in any sample of decayed wood, and such statistical methods as might be used to estimate it indirectly could only lead to rough approximations. It is therefore impracticable to apply corrections to the analytical data for material derived from mycelium. Comparison of the data in Table 5 with the analysis of sound oak wood (Table 1) reveals, however, that the material digested by the larvae is more akin in composition to wood than it is to *P. cryptarum* mycelium so that mycelium can, at most, constitute but a small part of the material digested.

There remains the possibility that fungal mycelium contains a component essential to the beetle, but which occurs in amounts too small to affect the analytical results in Table 5. In this connexion the N metabolism of the insect immediately suggests itself, but there is unfortunately no evidence to show whether *P. cryptarum* is a necessary intermediary for the modification of the wood nitrogen to suit the beetle. Little evidence appears to be available concerning the N metabolism of wood-eating insects as a whole, but Hopf [1937] has concluded that the *Lyctus* powder-post beetles can utilize the organic N of oak wood directly and it is possible that *Xestobium* can do the same, especially since the latter is known to develop, albeit slowly, in sound wood. It is noteworthy that the N requirements of *Xestobium* (Table 6) are quite small and that in the average sample of oak wood there is more than enough of this element to meet the requirements of all the larvae it could support.

The effect of previous fungal decay in wood on the economy of the Xestobium *larva from an energy standpoint*

Having elicited that the diet of *Xestobium* larvae reared in decayed wood consists essentially of wood components and not, to any recognizable extent, of fungus components, it is now necessary to determine the nature of the advantages which accrue to the insect as a direct result of passing its larval stage in decayed as opposed to sound wood. This question can best be approached from

the standpoint of the energy metabolism of the insect, since healthy continuity of the species must clearly depend upon the ability of the larva to derive sufficient energy from its food in order to provide for the succeeding stages of the insect in which further intake of energy from an external source is precluded. Of the total energy derived by the larva from its food, part is conserved as body tissue and part is dissipated in basal metabolism and the work metabolism involved in boring. Growth and development of the larva are only possible so long as the energy dissipated is less in amount than the total energy derived from the food and, other things being equal, they are at an optimum when this energy difference is greatest. Such being the case it is necessary, in view of the finding [Fisher, 1935] that the duration of the larval stage tends to become shorter as the extent of decay in the substrate increases, to determine whether decay renders the components of wood more readily available to the larva as a source of energy so that it is enabled to conserve energy at a greater rate when boring in decayed as opposed to less decayed or even sound wood. From the biological standpoint it would appear, from results about to be published, that fungal decay has such an effect although, as will be seen from the considerations which follow, the causal factors are more involved than might have been expected.

In Table 3 each loss in weight due to insect attack represents the food, and therefore the total energy, abstracted from the substrate by a group of larvae whilst the sum of the weight of frass and this loss in weight is the total weight of wood disintegrated by the group in order to obtain the food. When the loss in weight due to insect attack is expressed as a percentage by weight of the total wood disintegrated by any particular group of larvae a direct measure, which will hereafter be referred to as the "abstraction coefficient", is obtained of the amount of energy abstracted by the group per unit weight of wood disintegrated. It will be observed that the abstraction coefficient is independent of the number of larvae in any particular group and also of the time taken by the group to disintegrate any given weight of wood. Since all seven categories of decayed wood (Table 3) were subjected to beetle attack under the same conditions, direct comparison of the several abstraction coefficients is permissible. It is observed that, as fungal decay in the substrate increases, the numerical value of the abstraction coefficient decreases. This signifies that, over the range examined, the total amount of energy abstracted by the larva per unit weight of wood disintegrated decreases as the extent of fungal decay increases, unless it can be shown that the decline in the weight of food digested per unit weight of wood disintegrated is offset by an increase in the calorific value of the food. The calorific value of oak wood is given as 4390 cal./g. and of cellulose as 4150 cal./g. [Kollmann, 1936]. The calorific value of lignin is of the order of 5000 cal./g. It is unlikely that any component of oak wood has a calorific value higher than that of lignin. The calorific value of the larval diet can therefore increase with increasing extent of decay in the substrate if the proportion of lignin therein increases. If such were the case, a maximal calorific value would be obtained if and when the diet consisted solely of lignin. It is obvious, however, that so long as the abstraction coefficient (Table 3) is numerically greater than the percentage lignin content of the wood disintegrated by the larvae, the diet cannot consist solely of lignin. Moreover, in view of the analytical data in Table 5 it is concluded that a decline in the numerical value of the abstraction coefficient (Table 3) from 35·28 to 21·15 is not accompanied by any increase in the lignin content of the diet of sufficient magnitude to obviate a decline in the total amount of energy abstracted by the larvae, per unit weight of wood disintegrated. By inspection of the analytical data in Tables 1 and 5 it is deduced

that in wood which has lost up to 40 % of its weight by fungal decay the calorific value of the larval diet cannot be much, if any, greater than that of sound wood so that even in the unlikely event of a rapid increase of the lignin content of the diet in samples of wood decayed to losses in weight in excess of 40 % it can be seen that the total amount of energy abstracted from unit weight of badly decayed wood can never exceed that abstracted from moderately decayed wood. The above conclusion concerning the decline in the total amount of energy abstracted from unit weight of wood disintegrated as the extent of fungal decay in the substrate increases is therefore valid. Since the analytical data in Table 1 provide no indication of a decline in the dietetic value to *Xestobium* of oak wood as it becomes more decayed by *Phellinus cryptarum*, it must be concluded that the observed decline in the abstraction coefficient not only connotes an increase in the rate of boring which is not accompanied by a corresponding increase in the capacity of the larval gut to assimilate energy from the wood comminuted, but affords conclusive proof that the prime function of the fungus in this biological succession is *not* to modify the components of wood chemically so that they are more readily digestible by the larva. From this it follows that if the larva is to conserve energy at a greater rate in consequence of previous fungal decay in the substrate, it must do so chiefly in virtue of a combination of circumstances resulting from changes induced in the physical properties of the latter. Such being the case, attention is at once focussed on the effect of fungal decay on the mechanical strength of wood, it being taken for granted that, in view of the depletion induced in the strength of wood by decay, decayed wood offers less resistance to larval boring than less decayed or sound wood. It is fortunately unnecessary to speculate here with regard to the particular mechanical property or properties of wood involved in this boring activity, since depletion, albeit of varying order, is common to all of them during progressive fungal decay. The important point to note, however, is that, as the results of Cartwright *et al.* [1936] and of Scheffer [1936], in particular, indicate, the rate of decline in strength with progressive decay is greater than the rate of decline in density. Herein lies a possible advantage to the *Xestobium* larva from an energy standpoint, since in boring unit distance through decayed as opposed to less decayed wood it may abstract unit amount of energy with less effort and possibly in shorter time. Similarly by a consideration of the data of Scheffer [1936], which relate to a white rot having an effect on the composition of wood which is in all respects similar to that produced by *Phellinus cryptarum*, the authors conclude that the rate of decline in mechanical strength induced by progressive decay in the wood under investigation may at times be greater than the rate of decline in the larval abstraction coefficient over the range examined (Table 3). This factor, were it substantiated, would constitute a further advantage to the larva in that the disadvantage inherent in the observed decline in the abstraction coefficient would be to some extent offset by the diminishing effort required in the comminution of unit weight of wood. It is therefore obvious that if either or both of these possible advantages to the larva were in fact operative, the rate of conservation of energy by the larva as body tissue would increase. But in addition to such possible advantages one other factor affecting the energy metabolism of the larva must be considered. It has been indicated above that as the extent of fungal decay in the substrate increases, the rate of larval boring increases. The possibility therefore exists that provided that the larva maintains a sufficient rate of increase in its rate of boring as decay in the substrate progresses it can not only increase its rate of abstraction, but also its rate of conservation of energy.

Three means whereby the *Xestobium* larva may profit from an energy standpoint through living in decayed wood have been shown to be possible. All depend on depreciation of the mechanical strength of the substrate. How they are correlated, as correlated they must be, and which is the most important, cannot be deduced from the available experimental data, but since the biological observations already referred to indicate strongly that progressive fungal decay renders wood more and more suitable for the development of the *Xestobium* larva, the authors feel justified in emphasizing that predigestion of wood components by enzymic decomposition is not a prime factor, and in indicating the purely physical factors which either singly or in combination might account for the biological results.

The relationship between the destruction by Xestobium *of structural timbers in buildings and the presence therein of fungal decay*

It should be emphasized that the *Xestobium* frass examined by Campbell [1929], Ripper [1930] and Norman [1936] was in each case obtained from timbers which had been attacked *in situ*, and not from material infected under laboratory conditions as in the present study. Each of these authors established pronounced differences in composition between frass and sound wood and attributed substantial depletions in cellulose and pentosans entirely to the digestive processes of the insect.

The present investigation has revealed, however, that of the wood which it disintegrates the food of *Xestobium* may often represent but a small proportion and it is, therefore, inconceivable that the large losses of wood substance implied in the results of the above authors are attributable to the action of insects alone. There can now be little doubt that the "damaged" wood of Norman [1936] had undergone fungal decay prior to or contemporaneously with invasion by *Xestobium* and that the proportion digested by the latter was almost insignificant compared with that decomposed by fungi. Similar deductions must be made regarding the material examined by Campbell [1929] and by Ripper [1930]. Since a low cellulose content, a low pentosan content, a high lignin content and a high content of alkali-soluble matter are characteristic features of wood decomposed by fungi of the brown rot type it is deduced that fungi of this type were active in the timber from which Campbell [1929] and Norman [1936] obtained their respective samples of frass, as well as in one of the samples examined by Ripper [1930]. That this is somewhat more than a coincidence is shown by the following considerations.

The data in Table 7 were obtained from samples of frass taken from a series of 20 samples of oak wood selected statistically from a stock of infested material accumulated at this Laboratory from various buildings in England over a period of years. It is observed that in 16 out of 20 cases the percentage of frass soluble in 1 % NaOH under standard conditions is over 44. The corresponding figure for sound oak wood is known to vary between approximately 22 and 30 % so that from premises already adduced it can be concluded that in these 16 cases there has been fungal decay of the brown rot type in the wood from which the frass was produced and, moreover, that the extent of decay has varied considerably from piece to piece. In three cases the percentages soluble in alkali lie between 30 and 40 % and can only be taken to indicate that some form of decay was originally present. In the remaining case the percentage soluble in alkali (26·09 %) falls within the range for sound wood, so that the wood from which the frass was produced may or may not have been decayed by a white rot fungus. A more definite conclusion cannot be reached in these latter 4 cases because it has been

Table 7. *The action of 1% NaOH for 1 hr. at 100° on* Xestobium *frass taken from a series of samples of oak wood infested under natural conditions*

Sample no.	% Dry weight soluble in NaOH
1	45·41
2	53·78
3	37·86
4	77·72
5	55·68
6	53·73
7	55·44
8	50·40
9	46·47
10	60·73
11	51·00
12	48·25
13	39·42
14	54·01
15	44·91
16	57·54
17	50·90
18	32·73
19	59·59
20	26·09

found [Campbell, 1932] that the percentage soluble in alkali of wood decayed by a white rot fungus may vary from a value slightly greater to slightly less than that of sound wood. If the complete set of data in Table 7 be taken as statistically representative of the *Xestobium*-infested oak wood in England it must be deduced not only that such timber is usually more or less decayed, but that, in the majority of cases, the decay is of the brown rot type. It is a matter of experience that brown rots are more commonly found in oak timbers in buildings than are white rots. It is therefore indicated that there is no specific fungus which predisposes oak wood to *Xestobium* attack and that attack is not limited to wood which has attained any particular stage of decay.

Summary

1. The effect of decay by *Phellinus cryptarum* Karst. on the chemical composition of oak sapwood has been studied in detail. The fungus is shown to cause a white rot of the class in which all of the major components of wood are decomposed simultaneously.

2. The bearing of previous decay by *P. cryptarum* on the availability of oak sapwood to the death-watch beetle (*Xestobium rufovillosum* de G.) has been examined.

3. The food of *Xestobium* is shown to consist of extractives, cellulose, hemicelluloses and lignin and it is proved that there is no specific type of fungal decay which predisposes oak wood to attack by the insect.

4. Although decayed wood is the commonest habitat of *Xestobium* this circumstance does not depend on the relative abundance or availability of any particular food component resulting from fungal activity.

5. The wood digested by a group of insects when expressed as a percentage by weight of the wood disintegrated during boring by the same group is termed the "abstraction coefficient". The abstraction coefficient is independent of the number of larvae in any particular group and also of the time taken by the group to disintegrate any given weight of wood. The coefficient is accepted as a

direct measure of the amount of energy abstracted by the *Xestobium* larva per unit weight of wood disintegrated.

6. It is found that as the extent of fungal decay in the substrate increases the numerical value of the abstraction coefficient diminishes over the range of loss of 26–73 % of the dry weight of original wood caused by decay. This is cited as evidence that the prime function of the fungus in the biological succession is *not* to modify the components of wood chemically so that they are rendered more readily digestible by the *Xestobium* larva.

7. It is concluded that the depreciation in the mechanical strength of wood induced by fungal decay is the major factor responsible for the relatively more favourable development of the *Xestobium* larva in decayed as opposed to less decayed or sound wood.

8. Decayed wood offers less resistance to boring on the part of the larva than does sound wood. The fact that during progressive fungal decay the rate of decline in the strength of wood is greater than the rate of decline in density together with the possibility that the rate of decline in the larval abstraction coefficient is not as great as the rate of decline in the mechanical strength of the substrate constitutes an advantage to the *Xestobium* larva so far as its energy metabolism is concerned.

9. It is indicated that as the extent of fungal decay in the substrate increases, the rate of larval boring also increases. The possibility, therefore, exists that the larva may regulate its rate of boring so that as the mechanical strength of the substrate decreases it is enabled to increase its rate of abstraction and conservation of energy.

10. The relationship between fungal decay and *Xestobium* attack in structural timbers under natural conditions is discussed.

The work described above was carried out as part of the programme of The Forest Products Research Board and is published by permission of the Department of Scientific and Industrial Research. The authors desire to record their indebtedness to their colleagues Dr R. C. Fisher and Mr E. D. van Rest for much helpful criticism.

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167. ZYMOTIC FUNCTION OF THE PHARYNGEAL, THORACIC AND POST-CEREBRAL GLANDS OF *APIS MELLIFICA*

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(*Received 24 September 1940*)

ALTHOUGH work has been done in regard to the glandular function [Hoskins & Harrison, 1934] little is known concerning the specific activity of these glands in the bees engaged in the different parts of the economy of the hive. The work here described is concerned with such an investigation and an attempt to determine the comparative degree of intensity of the reaction.

The writer, possessing a colony of bees, had material always at hand and experiments were carried out at will.

The three types of bees examined were: (1) nurse bee, (2) foraging bee, (3) wax bee. They were collected in the following manner:

Nurse bee. The hive was opened and the nurse bees were picked off the comb.

Foraging bee. These were taken from the alighting board.

Wax bee. This presented some difficulty owing to the trouble in differentiation of the type. However, a comb was taken from the brood chamber and a space was left. In a few days there was a good supply of wax bees. For the experiments with wax bees, the glands were taken from the bees and the wax scales on the same bee were used for the same experiment.

Dissection. The bees were asphyxiated with chloroform except for post-cerebral gland experiments when paraldehyde was used. Pins were used to fasten the bee to the wax-dissecting trough and the dissection was done in citrated saline solution.

Pollen suspension. This was prepared by grinding the pollen from mixed wild flowers in an agate mortar until a fair degree of rupture was obtained. This was ascertained by microscopical examination. 0·01 g. pollen to 5 ml. sterile water.

Sucrose solution. 1% sterile solution.

Wax scales. From the wax bee.

The specific functions of the three types of glands in the three types of bees were determined. For this purpose the pharyngeal, thoracic and post-cerebral glands were dissected out of the nurse bee, foraging bee and wax bee, and these glands were each examined in accordance with the appropriate method described in later paragraphs in this paper.

	Nurse bee	Foraging bee	Wax bee
	Proteolysis test		
Pharyngeal gland	++	+	−
Thoracic gland	−	−	−
Post-cerebral gland	−	−	−
	Inversion test		
Pharyngeal gland	−	−	−
Thoracic gland	++	+++	+
Post-cerebral gland	−	−	−
	Lipoid test		
Pharyngeal gland	−	−	−
Thoracic gland	−	−	−
Post-cerebral gland	−	−	+++

Having thus determined the specific actions of the glands the degree of their comparative intensities was next attempted and recorded in the following paragraphs.

Pharyngeal glands

Proteolysis test. 1 ml. of the pollen suspension was intimately mixed with seven lobules of the pharyngeal glands of each of the three types of bees, viz. nurse, foraging and wax bees. The glands were well ground with a glass rod on the tube and then 1 ml. of pollen suspension was added. The whole was thoroughly mixed, incubated at 37° and tested at intervals for soluble protein with 5% phosphotungstic acid. The test was carried out as follows: a small drop was taken from the tube and put on a piece of black glass, the phosphotungstic acid solution was then brought to it and the junction of the two liquids was examined for a cloud or perhaps a precipitate.The examination was made visually and microscopically by means of reflected, oblique and polarized light and dark-ground illumination. Throughout the examinations the initial stage of protein precipitation was detected by means of dark-ground illumination. This detection was the precursor of a slight cloud that developed at a later time in the test. The slight cloud was taken as positive proteolysis and Table 1 is a record of the results obtained.

Table 1. *Pharyngeal gland plus pollen suspension*

	Proteolysis time in hr.		
Exp.	Nurse bee	Foraging bee	Wax bee
1	1·0	2·5	>3·0
2	1·75	2·0	>3·0
3	2·0	1·5	4·0
4	1·5	2·5	2·5
5	2·0	2·25	4·5
6	1·0	3·0	3·0
7	2·0	3·0	2·5
Average	1·6	2·4	3·2

Thus the order of maximum proteolytic activity of pharyngeal glands was: (1) nurse bee, (2) foraging bee, (3) wax bee.

A control gave a negative result: it was run as follows. 1 ml. of pollen suspension, without gland preparation, was incubated with the other tubes and subjected to the same tests. The result indicated that during the period of test there was no autolysis.

Thoracic glands

Inversion test. 3 ml. of 1% sucrose solution were intimately mixed with seven lobules of the thoracic glands of each of the three types of bee, nurse,

Table 2. *Thoracic glands plus sucrose solution*

	Inversion time in hr.		
Exp.	Nurse bee	Foraging bee	Wax bee
1	1·5	1·0	>3·0
2	2·5	0·75	>3·0
3	2·0	1·5	>3·5
4	3·0	1·0	3·0
5	1·5	1·5	2·75
6	2·5	1·0	2·5
7	1·75	1·75	>3·0
Average	2·1	1·2	Approx. 3·0

foraging and wax bees. They were then incubated at 37° and tested at intervals with Fehling's solution for reduction (Table 2).

3 ml. of the Fehling's solution, incubated alongside the other tests, indicated the absence of auto-reduction.

Order of maximum inverting activity of thoracic glands: (1) foraging bee, (2) nurse bee, (3) wax bee.

Post-cerebral glands

Lipoid test. The glands were dissected out from each of the three types and one lobule was used for three wax scales. The gland was taken from the same bee as the wax. The wax was pasted with the gland by means of needles under a binocular microscope. The mixture was then incubated at 37° for 3 hr. At the end of this time the melting-point of the treated wax scales was determined and found to be 68°. Previous M.P. determinations had shown that of the untreated wax scales to be 67–68°. The test was carried on for 4 hr. and the M.P. again determined and found to be 68°. A further series of tests was started and carried on for 8 hr., the M.P. again determined, and Table 3 is a record of the results.

Table 3. *Post-cerebral gland and wax after 8 hr. incubation*

Exp.		Nurse bee	Foraging bee	Wax bee
1	M.P. (68°)	68°	67°	64°
2	M.P. (68°)	67°	65°	63°
3	M.P. (67°)	67°	67°	64°
4	M.P. (67°)	66°	67°	65°

The M.P. of the mixture before the experiment is the figure in brackets. As one bee did not provide sufficient wax for a complete experiment the small deficiency had to be made up from another bee. For the dissection of the post-cerebral glands the bee was anaesthetized with paraldehyde. It was found that when chloroform was used there was a tendency for the gland contents to assume a granular condition. When these glands were used for the wax experiments they gave a negative result. Paraldehyde was then used and the gland contents were found to be homogeneous and considered suitable for the experiment. Wax untreated with gland preparation and incubated alongside the experiment was found to have M.P. identical with that determined before incubation. For this control test wax scales from the same bee were used to determine the M.P. before and after the incubation period.

Summary

The three types of glands, viz. pharyngeal, thoracic and post-cerebral, are specific in their actions. The latter vary in intensity in the three types of bee, viz. nurse, foraging and wax bees. Whilst the nurse bee shows the greatest proteolytic activity it is also possessed of an enzyme system of the nature of invertase; this factor suggests the potentiality of a dual role, with the preponderance in favour of proteolysis. Proteolytic activity is analogous with the foraging bee but in this case the invertase system is predominant and proteolysis secondary. The wax bee is of a type more specific than the nurse or the foraging bee since the enzyme system which is of the lipoid type is peculiar to the wax bee and the activities of the proteolytic and inverting systems are of a very low degree.

Throughout the text the term lipoid system has been used, but I do not think my determinations are sufficient to establish the presence of a lipase in the post-cerebral glands of the wax bee.

I wish to thank C. G. Butler, Esq., of the Bee Research Dept., Rothamsted, for his helpful criticism.

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168. SCLEROTIORINE, $C_{20}H_{20}O_5Cl$, A CHLORINE-CONTAINING METABOLIC PRODUCT OF *PENICILLIUM SCLEROTIORUM* VAN BEYMA

By TIMOTHY P. CURTIN and JOSEPH REILLY

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(*Received 4 September 1940*)

One of the five strains of *Aspergillus terreus* [Raistrick & Smith, 1936] yields two metabolic products, containing chlorine: geodin, $C_{17}H_{12}O_7Cl_2$, and erdin, $C_{16}H_{10}O_6Cl_2$. These compounds, crystallizing in fine yellow needles and melting with decomposition, are the first recorded instances of chlorinated metabolic products of the lower fungi. More recently, the isolation of griseofulvin, $C_{17}H_{17}O_6Cl$ [Oxford *et al.* 1939] and caldariomycin, $C_5H_8O_2Cl_2$ [Clutterbuck *et al.* 1940], chlorinated products of *Penicillium griseofulvum* and *Caldariomyces fumago*, respectively, has been described.

The subject of the present communication, and to which we propose to give the name *sclerotiorine*, $C_{20}H_{20}O_5Cl$, closely resembles the above in crystalline structure but melts without decomposition or formation of a sublimate at 206–7° [Curtin & Reilly, 1940].

The strain of *P. sclerotiorum* used was obtained from the Centraalbureau voor Schimmelcultures and shows under certain well-defined conditions of temperature, mycelial pigmentation, ranging from yellow through orange to red, which colour is particularly apparent in the actual sclerotia. Sclerotiorine is very slightly soluble in cold dil. Na_2CO_3 and $NaHCO_3$ solutions and is obtained in yield of about 2% of the dry mycelium weight.

Experimental

Culture

The organism was first isolated from the air by Prof. Boedijn in Buitenzorg, Java, in 1935 and sent to the Centraalbureau voor Schimmelcultures in May 1936 to be described by van Beyma [1937].

At the optimum temperature, the mould forms masses of orange-red sclerotia, which obscure the blue-green conidia while the reverse is also an intense orange-red. Thus, at 25° pigmentation begins on malt agar in 3–4 days. If, however, the cultures are grown at room temperature the formation of the conidia is almost complete and the appearance of the sclerotia is greatly delayed. The red colouring matter of the organism is soluble in alcohol and ether, giving an orange-yellow solution.

P. sclerotiorum fits Thom's classification of the Monoverticillata stricta.

Cultural conditions

The culture medium used throughout was the well-known Czapek-Dox solution, containing KCl as the sole source of chlorine (glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·1 g., and distilled water to 1000 ml.).

This medium was distributed in 100 ml. amounts in a batch of 100 20-oz. flat bottles, plugged with cotton wool, sterilized by steaming on each of three consecutive days and sown with a spore suspension of *P. sclerotiorum*, grown for 10 days on malt-agar. The bottles were incubated on the horizontal at approximately 25° in the dark for 24 days. Growth in light gives much inferior mycelium and pigment production.

Mycelium formation became apparent in 2 days and in 4 days a faint orange tint appeared in the upper side of the mycelial felt while the reverse was a bright yellow. After 10 days, the reverse was intensely orange-red and the surface of the mat was salmon pink, interspersed with small white spots. The mixed contents of three bottles were taken every 3 days and tested for *p*H.

Days of incubation	*p*H
0	4·2
3	3·7
6	3·2
9	3·6
12	5·3
15	5·7
18	6·2
21	6·7
24	7·1

After 24 days, the mycelium was strained off and the light golden-yellow medium discarded. When pressed and dried at 50–60° *in vacuo*, the mycelium from 79 bottles weighed 124 g., which corresponds to a yield of 15·5 g. of dry mycelium per initial litre of substrate.

Preparation of sclerotiorine

The finely ground mycelium was completely extracted with light petroleum (B.P. 60–80°) in a Soxhlet apparatus; 0·85 g. of an orange-coloured precipitate was filtered off from the red extract and mycelium, though still coloured, could not be extracted with alcohol. On removal of the light petroleum by distillation, a further 1·60 g. of crude sclerotiorine separated out. At this stage the product melted at 197–201°.

The deep red residue remaining after the complete removal of the light petroleum did not crystallize, and when dissolved in alcohol and diluted with water it gave a cloudy golden orange solution, from which there was no separation of solid material on long standing.

Pure sclerotiorine. The crude colouring matter was recrystallized several times from absolute alcohol and the final product, very fine hair-like yellow needles, melted sharply at 206–207°.

(Found: C, 64·09; H, 5·43 %; Cl, 9·47 %; OCH_3, nil. Mol. wt. 364. $C_{20}H_{20}O_5Cl$ requires C, 63·92; H, 5·33; Cl, 9·45 %. Mol. wt. 376).

Properties of sclerotiorine

(1) Sclerotiorine is insoluble in water but is soluble in most organic solvents.

(2) It dissolves sparingly in cold NaOH, dil. Na_2CO_3, $NaHCO_3$ and NH_4OH solutions. In the latter, it is slightly more readily soluble. The following reactions are noted with the various solutions.

(*a*) The solution in NaOH, yellowish brown, changes to a murky yellow with acid.

(*b*) In Na_2CO_3 and $NaHCO_3$ solutions, the golden yellow coloration shows little change on acidification.

(*c*) The wine-red solution in NH_4OH acidifies to golden yellow, the change being reversible. Sclerotiorine is therefore an indicator and it must be noted that, at the turning point, the solution takes on a cloudy appearance.

(3) With an alcoholic solution of the pigment, NaOH, NH_4OH and Na_2CO_3 give red colorations, but with $NaHCO_3$ the solution remains yellow with a separation of the compound.

(4) Sclerotiorine in alcoholic solution reduces acidified $KMnO_4$, but is not itself reduced by $Na_2S_2O_4$ solution.

(5) Traces of chloride can be detected on prolonged boiling of the pigment with distilled water. On heating with NaOH and acidifying with HNO_3, a precipitate is obtained on the addition of $AgNO_3$ solution.

(6) On addition of alkali to the orange-yellow alcoholic solution, a red coloration is obtained and this is decolorized by Zn dust and heating.

(7) The alcoholic solution does not give a coloration with alcoholic $FeCl_3$.

(8) The addition of much water to the alcoholic solution precipitates the pigment.

(9) Sclerotiorine does not show oxonium salt formation with anhydrous HCl in absolute ether.

(10) With conc. H_2SO_4 the compound yields a bright red-orange colour, changing to yellow on dilution.

(11) Sclerotiorine sublimes in a high vacuum at 175–180°. The sublimate of microscopic needles melts unchanged at 206–207°.

(12) In alcoholic solution, sclerotiorine does not liberate iodine from acidified KI solution. It is not, therefore, a *p*-quinone.

On dilution of the alcoholic mother liquors of crystallization of sclerotiorine, a red compound, melting at approximately 150°, separates out in yield too small for examination.

The properties of sclerotiorine are being further investigated.

We desire to thank Prof. A. Robertson for his liberal advice and guidance and Mr G. Fitzgerald, M.Sc., for his assistance in maintaining the cultures.

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169. THE CONSTITUTION OF ARACHIDONIC ACID (PRELIMINARY COMMUNICATION)

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(*Received 2 October 1940*)

Arachidonic acid is widely distributed in the animal body and its presence has been shown to be essential for the maintenance of the normal health of the rat. In rats which have suffered for many months from the fat deficiency disease first described by Burr & Burr [1930] the liver was found to be entirely free from this acid and only very small amounts were detected in other parts of the body. It was, however, retained if linoleic or arachidonic acid itself was included in the diet of the rats. The conclusion was drawn by Nunn & Smedley-MacLean [1938] that the rat can probably synthesize arachidonic acid using linoleic acid as its starting material. At this time all that was known as to the structure of arachidonic acid was that it contained a normal chain of 20 carbon atoms since it yielded arachidic acid on reduction [Bosworth & Sissons, 1934], that four double bonds were present and that, from the normal iodine value, these were unconjugated. We were therefore anxious to determine the position of the double bonds in the chain and to compare the structure of this acid with those of linoleic acid.

Shinowara & Brown [1940] have, however, recently published some preliminary results on the structure of this acid and suggested a formula though they emphasize that it is only tentative. The determination of the diene number of methyl arachidonate is interpreted by these authors as indicating the presence of about 5% of conjugated bonds derived either from the presence of a small proportion of an isomeric ester or from a rearrangement of the ester under the conditions of the diene determination. However, in the bulk of the acid examined, the bonds were not conjugated. The methods of oxidation used by these authors were ozonolysis and oxidation with permanganate in acetone solution. We, on the other hand, have used the action of an aqueous alkaline permanganate solution and have arrived at entirely different conclusions from those drawn by Shinowara & Brown. We should have preferred to repeat and extend our experiments before publishing our results but in view of the communication of these authors and of the postponement of our experiments necessitated by present conditions, we have decided to publish them.

Shinowara & Brown, with their methods of oxidation, did not detect any aldehyde higher than acetaldehyde, nor did they detect oxalic acid but they obtained indications of adipic and succinic acids and concluded that a double bond existed between the 18th and 19th carbon atoms and that the group $:C.CH_2.C:$ characteristic of linoleic and linolenic acids was not present in the molecule. We on the other hand deduce from our findings that the terminal chains of ten carbon atoms are similar in structure in both arachidonic and linoleic acids and that certainly three and almost certainly four of the groups $:CH.CH_2.CH:$ occur in the arachidonic molecule.

We used as our starting material the acid prepared by debromination of the ether-insoluble arachidonic octabromide obtained by brominating the fatty acids of ox suprarenal glands and extracting the product with ether in a Soxhlet apparatus following the directions given by Brown [1928]: I.V. = 334 (calc. 334·2). In order to avoid rearrangement of the position of the double bonds, the acid was not distilled. Farmer & Van den Heuveland [1938], in working with the cod-liver oil acids, had found evidence of increased molecular refraction appearing after the methyl esters had been distilled even at low pressure; but there was no increased refraction when the process of "molecular distillation" was used.

Oxidation by alkaline permanganate

3·18 g. arachidonic acid were dissolved in a solution of 30 g. KOH in 2 litres water and to this were added 50 g. $KMnO_4$ dissolved in 1·5 litres water. Both solutions were cooled to 0° and the $KMnO_4$ solution added to the well-stirred soap solution during rather less than 10 min. The reaction mixture which at the end of 2·5 hr. at room temperature was still alkaline and contained excess of permanganate was decolorized by passing in SO_2 and 60 ml. conc. H_2SO_4 were diluted and added. The mixture was then steam-distilled; the distillates containing SO_2 and volatile acids were neutralized, evaporated to dryness and the residual aqueous solution extracted 25 times by ether.

Volatile acids. The salts of the volatile acids were acidified and again steam-distilled, the distillates being now free from sulphite or sulphate. The distillate was collected in successive portions of 30 ml. and the equivalents of the acids present in these fractions determined by comparing the amount of 0·1 *N* NaOH required for neutralization with the dry weight of sodium salt obtained. The distillation was continued until 30 ml. of distillate required less than 1 ml. 0·1 *N* NaOH for neutralization. The first fraction was distinctly milky, the equivalent of its acid being 102·2; the acid equivalents of the next three fractions lay between 96·6 and 98·7. The final 635 ml. of distillate contained acid of average equivalent 60 but in the final 60 ml. of this distillate one-third of the acidity was due to formic acid. The acid of the first fractions (equiv. 102) smelt strongly of valeric acid. Since, however, lower acids were present in the mixture, the presence of a higher homologue was not excluded, the most probable interpretation being the presence of valeric with possibly some hexoic acid. A test tube experiment showed that under the conditions of our experiment if sodium valerate was left with alkaline permanganate some reduction takes place, so that some amount of lower acid will in any case probably be formed.

0·12 g. Na salt prepared from the first fraction was boiled under a reflux for 1·5 hr. with 0·3 g. *p*-bromophenacyl bromide in aqueous alcoholic solution and rapidly cooled; 0·18 g. crystals (M.P. 46–50°) was obtained, and the M.P. raised by recrystallization to 58–60°. *p*-Bromophenacyl butyrate, valerate and hexoate were prepared for comparison. M.P.: butyrate 61·2°; valerate 72–73·5°; hexoate 70–71·5°. Crystals prepared from the 2nd and 3rd fractions melted at 55–57°.

Mixtures of crystals (M.P. 58–60°) with butyrate (M.P. 61·2°): softened at 45°, melted at 49–50°; valerate (M.P. 72·3°): softened at 63°, melted at 71·2°; hexoate (M.P. 70·5–71·5°): softened at 65°, melted at 68–70°. These results seemed to exclude the presence of butyric acid and suggested the possible presence of a mixture of hexoic and valeric acids. A mixture of *p*-bromophenacyl-valerate and -hexoate softened at 63° and melted at 67°.

By further crystallization from aqueous methyl alcohol the melting-point of the crystals was raised to 55–60°. Two kinds of crystals were present, transparent plates and opaque clumps. When the transparent plates were separated as far as

possible, these melted from 65 to 70°; mixed with *p*-bromophenacyl valerate, M.P. 73°, they softened at 65° and melted completely at 71–72°; with the hexoate, M.P. 70–71°, they softened at 55° and melted at 60–65°. From a consideration of the equivalent, the behaviour of the *p*-bromophenacyl ester and the odour, the chief acid present is certainly valeric, and it is most probably accompanied by some hexoic acid.

Identification of dibasic acids. The residual aqueous solution was extracted with ether 25 times; this extract gave no precipitate with a solution of 2:4-dinitrophenylhydrazine; ketonic groups were therefore absent.

The ethereal solution of the first 14 extracts contained 2·59 g. solid of which 0·44 g. separated as sparingly soluble white crystals losing 28·24 % of their weight in the desiccator; M.P. 188–189° (anhydrous). $(COOH)_2\ H_2O$ loses 28·5 %; anhydrous oxalic acid, M.P. 189°. A small amount of the crystals washed with ether on a tile and dried gave an equivalent by titration of 49·2. The equivalent weight of anhydrous oxalic acid is 45. A solution of the sodium salt gave with $CaCl_2$ solution and ammonia the white flocculent ppt. characteristic of oxalic acid.

The total amount of $(COOH)_2$ present was therefore calculated as follows. From the ether-soluble fractions 1–14, 0·316 g. anhydrous oxalic acid was isolated. A portion of the remainder was titrated with $N/10$ $KMnO_4$ in acid solution and the amount of oxalic acid estimated. From similar titrations carried out on the subsequent ether extracts the total amount of oxalic acid was computed as 1·9 g., representing approximately 2 molecular proportions of oxalic acid for each molecule of arachidonic acid oxidized.

The residual aqueous liquid after repeated ether extraction was then treated with $N/10$ $KMnO_4$ in acid solution to remove any remaining oxalic acid. This liquid, however, reacted only slowly with the permanganate using in all 16·0 ml. corresponding with a possible maximal amount of 0·07 g. oxalic. The residual aqueous solution from the first 14 fractions which had been treated with acid permanganate to remove all oxalic acid present contained about 0·5 g. of some substance other than oxalic acid. It was therefore concentrated to a small bulk and ten times extracted with ether. 0·69 of somewhat sticky crystals was obtained. From this, by recrystallization from water, 0·1 g. crystals melting at 142–150° was obtained. This suggested the presence of adipic acid, M.P. 154°, but the mixed melting point of the crystals with a specimen of adipic acid (M.P. 149–150°) was 125–135°, with suberic acid (M.P. 140°), 122–130°. The crystals were therefore neither adipic nor suberic acid. By recrystallization from ether the M.P. was raised to 160–165° and a mixed melting point with succinic acid (M.P. 186–187°) was 165–180°, on remelting 170–178°. No evidence was obtained of the presence of any dibasic acid other than oxalic and succinic.

The aqueous residue from fractions 15–19 was added to the residual aqueous solution from fractions 1 to 14 and extracted with ether but no satisfactory product was obtained.

The final ether extracts were not treated with $KMnO_4$: the solid residue was extracted with benzene; after washing on a porous tile 14 mg. white needles were obtained (M.P. 85–89°), mixed M.P. with glutaric acid (M.P. 92–95°), 88–91°.

The benzene-insoluble residue melted at 95–105°: it dissolved in ether and from this solution two sets of crystals were separated; these were picked out by hand and consisted of

(*a*) 0·21 g. of well-defined cubes, M.P. 90–100°, equiv. by titration 65. These crystals were extracted with $CHCl_3$ and the extract filtered; the solution deposited crystals (M.P. 95–98°), mixed with glutaric acid (M.P. 93°), M.P. 71°. These were probably hydrated oxalic acid.

(*b*) A very small quantity of needles, M.P. 180°. These were probably succinic acid.

The final residual liquid which had been repeatedly extracted with ether and then treated with acid permanganate was precipitated with barium chloride with the object of separating an acid-soluble barium salt from the mass of $BaSO_4$ thrown down. This attempt was not successful.

From 3·14 g. arachidonic acid, there is therefore evidence of the formation of approximately 2 molecular proportions of oxalic acid, of succinic and valeric with probably some hexoic acid, of lower volatile acids and of a very small amount of glutaric acid.

Since from determinations of the iodine value and of the diene number arachidonic acid probably does not contain conjugated double bonds, a chain of 11 carbon atoms is the shortest that will include the four double bonds without conjugation, i.e. inserting one methylene group between each pair of double bonds. The presence of valeric acid with probably small amounts of hexoic acid, indicates a terminal *n*-amyl group attached to the ethylene group at one end of the chain of unsaturated linkages; the valeric acid would be formed if the ethylene group were oxidized to oxalic, the hexoic acid by fission of the double bond. If the terminal ethylene group nearest to the carboxyl were oxidized to oxalic acid, we should be left with succinic acid formed from carbon atoms 1–4. This structure seems confirmed by the isolation of the small amount of glutaric acid, which must have come from fission of the terminal group at the carboxyl end of the chain. The formula of arachidonic acid would therefore be regarded as

$$CH_3(CH_2)_4CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2CH{:}CH.(CH_2)_3.COOH.$$

This formula would agree well with the formation of arachidonic from linoleic acid since the chain of ten terminal carbon atoms is identical in the two compounds, these two double bonds representing the 9:10 and 12:13 positions in the 18 C chain. If we suppose that the lengthening of the chain takes place by the condensation of the carboxyl group of the C_{18} acid with some compound such as pyruvic acid or acetic acid, unsaturation must then occur in the 6:7 and 3:4 positions in the C_{18} chain or in the 8:9 and 5:6 positions in the C_{20} chain.

Since Shinowara and Brown used different methods of oxidation it is difficult to compare their results with ours. Using oxidation by permanganate in acetone solution they were unable to identify any oxalic acid. They agree with us in obtaining evidence of the presence of succinic acid which they confirmed by the analysis of the Ba salt. They obtained some evidence of the presence of an acid with M.P. 154°, equiv. 69·9 (adipic acid has M.P. 151–153°, equiv. 73) which they regarded as adipic acid. As described above, we found an acid with M.P. 142–150° which we thought might be adipic acid but this was disproved by a lowering of about 20° when the mixed melting point was taken. Shinowara and Brown identified acetaldehyde in an ozonolysis experiment; possibly this might originate from the oxidation of a $:CH.CH_2CH:$ group but the large proportion of volatile fatty acids with five and six carbon atoms obtained by oxidation with alkaline aqueous permanganate clearly indicates that the terminal ethylene linkage is not in the 17:18 position in the arachidonic chain. The formula suggested by Shinowara and Brown is not in accordance with the results of the oxidation with alkaline aqueous permanganate.

It may also be noted that in work on the oxidation of the hydroxy derivatives of linoleic and linolenic acids Green & Hilditch [1937] have shown that whereas in the oxidation of the dihydroxy derivatives of the monoethylenic acids the main fission is into oxalic acid and acids containing eight carbon atoms, with the tetra-

and hexa-hydroxy derivatives only 20 % of the acid is broken up in this manner. In the oxidation of arachidonic acid at least 50 % of the ethylene groups are separated as oxalic acid.

Summary

Oxalic, succinic, acetic, valeric with probably some hexoic and small amounts of glutaric and formic acids have been identified among the oxidation products of arachidonic acid with alkaline permanganate.

A formula is suggested in which the ten terminal atoms are similar in structure to those of linoleic acid.

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170. STUDIES OF CALCIUM AND PHOSPHORUS METABOLISM IN RELATION TO THE CHEMICAL STRUCTURE OF BONE

2. EXPERIMENTS WITH MOULTING BIRDS

By CYRIL TYLER

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(*Received 23 August 1940*)

In a previous paper, Tyler [1940] has shown that there is a reciprocal relationship between changes in the Ca of the $Ca_3(PO_4)_2$ of bone (designated Ca_P) and changes in the residual Ca of bone (designated Ca_R) for laying birds, and also that it is the Ca_R which is chiefly concerned with the fluctuations of bone Ca during laying. In the experiments from which these results were obtained two other birds moulted and it is now proposed to discuss the bone changes for these two birds. The experiments were described in the preceding paper and it is only necessary to add that the bird C1 received calcium carbonate as the calcium supplement and that the bird G1 received calcium gluconate.

Results

Table 1 gives the relevant data regarding the two birds. The balance data for the 4 days when G1 was laying are not truly representative, for no figures were available for one day out of the 4 and therefore they will not be considered

Table 1

Length of period days	Bird	No. of eggs laid	% Ca in food	% P in food	Ca : P of food	Ca balance over period	P balance over period	Ca : P of balance
10	C1	4	1·68	0·40	4·20	−1·09	0·37	—
25	C1	Moult	1·68	0·40	4·20	−8·99	−0·37	24·29
4	G1	2	1·65	0·32	5·16	−0·88	0·45	—
31	G1	Moult	1·65	0·32	5·16	6·19	−0·51	—

in that form; however, the daily figures have some value and these will be considered. As in the case of the laying birds, the Ca : P ratios of the total Ca and P balances do not approach 2·2 : 1, which is the value for normal bone, and in fact in the case of C1 in lay and of G1 in moult a positive value of one corresponds to a negative value of the other. This being the case, the chemical composition of the bone must be changing and calculations of Ca_P and Ca_R were therefore made to find out whether these values give any clue as to bone changes. The methods of calculation and a justification of such a calculation are given in the previous paper. It was there pointed out that such a calculation was not valid if the bird was losing or gaining considerably in weight, for this reason it has only been possible to calculate values for the very short laying periods for each bird and for the moulting period of bird G1. The bird C1 lost too much weight during the moult (355 g.) for the calculation to be of value.

Table 2. *Bird C1, 10 days in lay; Ca_P and Ca_R lost or gained by bone, the retention of Ca (Ca_A) and egg shell Ca (Ca_S) (g.)*

	Laying days				Non-laying days		
Day	Ca_P	Ca_R	Ca_A	Ca_S	Ca_P	Ca_R	Ca_A
1	—	—	—	—	+0·00	+0·20	+0·20
2	—	—	—	—	+0·46	+0·07	+0·53
3	—	—	—	—	+0·23	+0·53	+0·76
4	+0·42	−1·51	+0·46	1·52	—	—	—
5	—	—	—	—	−0·35	+1·04	+0·69
6	+0·37	−1·19	+0·82	1·59	—	—	—
7	—	—	—	—	−0·58	+1·02	+0·44
8	+0·41	−1·17	+0·98	1·69	—	—	—
9	—	—	—	—	−0·46	+0·52	+0·06
10	+0·19	−1·29	+0·71	1·76	—	—	—
Sum	+1·39	−5·16	+2·97	6·56	−0·70	+3·38	+2·68
Mean	+0·35	−1·29	+0·74	1·64	−0·12	+0·56	+0·45

Table 3. *Bird G1, 4 days in lay; Ca_P and Ca_R lost or gained by bone, the retention of Ca (Ca_A) and egg shell Ca (Ca_S) (g.)*

	Laying days				Non-laying days		
Days	Ca_P	Ca_R	Ca_A	Ca_S	Ca_P	Ca_R	Ca_A
1	+0·33	−1·25	+1·02	1·89	—	—	—
2	—	—	—	—	+0·54	+0·34	+0·88
3	—	—	—	—	Omitted because of incomplete data		
4	−0·02	−0·82	+1·05	1·84	—	—	—
Sum	+0·31	−2·07	+2·07	3·73			
Mean	+0·16	−1·04	+1·04	1·87			

Table 4. *Bird G1, 31 days in moult; Ca_P and Ca_R lost or gained by bone, and the retention of Ca (Ca_A) (g.)*

Days	Ca_P	Ca_R	Ca_A	Days	Ca_P	Ca_R	Ca_A
1	+0·25	+0·30	+0·55	16	−0·19	+0·60	+0·41
2	+0·48	−0·23	+0·25	17	+0·12	+0·37	+0·49
3	−0·10	+0·54	+0·44	18	+0·19	−0·27	−0·08
4	−0·27	+0·47	+0·20	19	+0·14	+0·24	+0·38
5	+0·08	+0·33	+0·41	20	−0·02	+0·51	+0·49
6	−0·35	+0·34	−0·01	21	+0·02	+0·47	+0·49
7	−0·23	+0·46	+0·23	22	+0·06	+0·24	+0·30
8	−0·37	+0·16	−0·21	23	−0·17	+0·37	+0·20
9	−0·37	+0·18	−0·19	24	0·00	+0·26	+0·26
10	−0·12	+0·05	−0·07	25	+0·15	+0·31	+0·46
11	−0·08	+0·22	+0·14	26	−0·04	−0·38	−0·42
12	−0·14	+0·36	+0·22	27	+0·02	−0·06	−0·04
13	−0·29	+0·35	+0·06	28	+0·29	−0·09	+0·20
14	−0·19	+0·21	+0·02	29	0·00	+0·55	+0·55
15	−0·14	+0·21	+0·07	30	+0·04	+0·06	+0·10
				31	+0·29	0·00	+0·29
				Sum	−0·94	+7·13	+6·19
				Mean	−0·03	+0·23	+0·20

The first day of the moult corresponds to the 5th day of full period.

Tables 2 and 3 give the values obtained during lay and Table 4 those obtained during the moult.

Considering first the few laying days of the birds it will be seen that the changes in Ca_P were small compared with those for Ca_R. The limitation of the total loss of Ca from the bone to a maximum value of about 1 g. is clearly seen

on all 4 days in the case of bird C1 and on the one day it was necessary in the case of G1; in each instance a large loss of Ca_R was partly counteracted by a gain of Ca_P. With regard to non-laying days C1 shows fairly small changes of Ca_P, some negative and some positive, but the large gains of Ca_R are only noticeable on 2 days of the period. The bird G1 had only one non-laying day but on that day there was not the usual relatively large gain of Ca_R. However, the findings for birds laying immediately before a moult are in general agreement with the findings for normal laying birds already described in the previous paper, namely, relatively large gains of Ca_R on non-laying days and relatively large losses on laying days with relatively small fluctuations of Ca_P, some being negative and some positive.

Turning to the moulting period for the bird G1 (Table 4) the same daily fluctuations in Ca_P are to be noted as for laying birds, with a loss as the final result; there were, on the other hand, almost consistent gains of Ca_R over the whole period of the moult which resulted in the bird storing 7·13 g. Ca as Ca_R

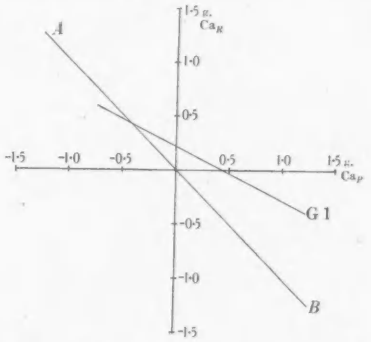

Fig. 1. Regression line of Ca_R on Ca_P for the moulting bird G1. *AB* represents the calcium equilibrium line. To the N.E. is a total calcium gain; to the S.W. is a total calcium loss.

over the 31 days. On calculating the correlation coefficient for Ca_P and Ca_R changes during the moult a value of $r = -0{\cdot}4309$ for 31 pairs of readings was found; this negative value is highly significant. The regression line representing the regression of Ca_R on Ca_P within the limits of the experimental values of Ca_R is shown in Fig. 1.

Discussion

From the results obtained it appears that the changes in Ca_P and Ca_R which take place in a laying bird just before moulting tend to be of a similar nature to the changes taking place in a bird in full lay but the correlation coefficient for Ca_P and Ca_R is not significant. The paucity of readings must however not be overlooked. In the case of the moulting bird G1 the changes in Ca_P and Ca_R were similar, but not as great in magnitude as those taking place in laying birds on non-laying days. A reciprocal relationship did hold between Ca_P and Ca_R for this moulting bird and a study of the regression line shows it to be similar to the ones obtained for laying birds on non-laying days [Tyler, 1940], except that it crosses the "balance" line within the region studied and not at some theoretical point beyond the range of experimental values.

Thus the Ca_P and Ca_R relationships already noted for four laying birds hold good for one moulting bird also. These relationships are such that a bird can show concomitant gains or losses of Ca_P and Ca_R or gains of one with losses of the other, and this makes it possible for the bones of a bird under differing conditions to show gains or losses of both total Ca and total P or gains of total Ca with a loss of total P or *vice versa*. By such means a bird is more able to accommodate itself to the calls made upon its bones than if concomitant gains or losses of Ca and P were alone possible.

Summary

The changes in the Ca of the $Ca_3(PO_4)_2$ of bone and the residual Ca of bone of two birds laying just prior to moulting and of one of the birds in moult are discussed. It is shown that these changes are substantially similar to the changes taking place in birds in full lay.

The reciprocal relationship between the changes of Ca_P and Ca_R which was shown to exist for four laying birds in a previous paper is found for one moulting bird.

The writer wishes to thank the University of Leeds under whose auspices the work was carried out.

REFERENCE

Tyler (1940). *Biochem. J.* **34**, 202.

171. ANALYSIS OF PROTEINS

12. DEPHOSPHOCASEOSE OR DEPOCASEOSE

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(*Received 20 August 1940*)

THE phosphorus of caseinogen is completely separated by the action of 1% NaOH at 37° in 24 hr. [Plimmer & Bayliss, 1906; Rimington & Kay, 1926]. Acidification of the solution precipitates a product called dephosphorized casein by Rimington & Kay [1926; Rimington, 1927] and dephosphocasein or depocasein by Plimmer & Lawton [1939], who made various analyses of its component amino-acids. Dephosphocasein represents about 60% by weight of the caseinogen (55% of the N). The remainder has now been found to consist mainly of primary proteose, dephosphocaseose or depocaseose with much smaller amounts of secondary proteose and peptones. This communication deals with the composition of depocaseose with some further analyses of depocasein.

Experimental

Distribution of the nitrogen after 24 hr. alkaline action

Hammarsten casein has been used in these experiments.

In preparing depocaseose it was deemed essential to avoid the presence of sodium acetate in the solution. Repetition of the conditions for maximal precipitation of depocasein by acetic acid showed that this occurred at *p*H 4·2. The precipitation is effected with HCl, using as indicator bromophenol blue which is just blue at this *p*H. It was first desired to study the nitrogen distribution of the products.

10 g. casein (8·93 g. dry protein) were dissolved and brought to 502 ml. with 1% NaOH (*N*/4). 2 ml. contained 5·23 mg. N. The remaining 500 ml. (1·32 g. N) were kept at 37° for 24 hr. and air was drawn through the solution to remove NH_3 originating from amide-N. To the solution, brought to 535 ml. (1·284 g. N) conc. HCl was carefully added with constant stirring to *p*H 4·2. Next day the depocasein was filtered off and washed with water. The filtrate and washings were brought to 890 ml. (0·61 g. N), warmed and saturated with recrystallized $MgSO_4,7H_2O$ (60 g. per 100 ml.) and kept overnight. The precipitate of primary proteose, depocaseose, was filtered off and the filtrate, made up to 1375 ml. (0·178 g. N), was saturated with crystals of $ZnSO_4$ with slight warming. After standing, the small precipitate of secondary proteose was filtered off. The filtrate (1370 ml., 0·145 g. N) was treated with 50 ml. of 20% phosphotungstic acid in *N*/2 HCl. The white flocculent precipitate of peptone was filtered off; the residual nitrogen in the solution was 0·027 g. In a second determination the depocaseose was thrown out by half-saturation with $ZnSO_4$. The results of these two experiments

with the N removed at each precipitation calculated as percentage of the total N taken are as follows:

	Exp. I		Exp. II		Mean
	g.	%	g.	%	%
Total N	1·320	—	1·330	—	—
NH_3-N	0·036	2·9	0·070	5·2	4·0
Depocasein-N	0·674	52·0	0·704	53·4	52·8
Depocaseose-N	0·433	33·0	0·403	31·2	32·1
Secondary proteose-N	0·033	2·5	0·051	4·7	3·6
Peptone-N	0·118	8·3	0·085	6·5	7·4
Residual N (amino-acids or reagents)	0·027	2·0	0·017	1·3	1·7

The action of 1 % NaOH on caseinogen at 37° thus yields two chief products, depocasein and depocaseose. Only 1/10 of the original protein-N consists of secondary proteose and peptone, mainly the latter.

Prepration of depocasein and depocaseose

Several smaller scale experiments were made to investigate the isolation of depocaseose free from contaminating salt and in a granular form. Half-saturation of the filtrate from depocasein with $(NH_4)_2SO_4$ was preferred on account of its easier removal by means of $Ba(OH)_2$; $BaSO_4$ was filtered off and NH_3 removed by evaporation *in vacuo*. Excess Ba ions were removed exactly with H_2SO_4. As first precipitated depocaseose dissolves slowly, yet completely, in water. It is insoluble in absolute alcohol, but about 60 % of the material dissolves in 75 % alcohol. When a concentrated solution is poured into 10 vol. of absolute alcohol a large proportion is thrown out in granular form which can be filtered off and dried by successive changes of absolute alcohol.

Large scale preparations were carried out with several batches of 40 g. casein which were placed in Winchester bottles with 250 ml. of 2 *N* NaOH and diluted to 2 litres with water. On shaking for a short time the casein dissolved. After warming to 37° the bottles were put in an incubator for 24 hr. The alkaline solutions were transferred to beakers and conc. HCl slowly run in with vigorous stirring until a precipitate just appeared; the depocasein was completely thrown out by adding small quantities of *N* HCl to *p*H 4·2. The precipitate, at first white and flocculent, agglutinated and adhered to the beaker. Next day the combined precipitates from 12 or 16 portions were filtered off, redissolved in *N* NaOH and reprecipitated at *p*H 4·2 by cautious addition of *N* HCl. The precipitate was washed with water and dried with successive changes of absolute alcohol and finally *in vacuo* over H_2SO_4. The combined filtrate and washings (about 30 litres) were concentrated *in vacuo* to about 1/3 vol., at which concentration frothing became troublesome, and the solution was half-saturated with the required amount of $(NH_4)_2SO_4$ crystals (400 g. per litre) and kept overnight. The depocaseose first settled out as a white flocculent precipitate, but on standing became a crusty mass slightly brown in colour. It was filtered off, redissolved in the minimum of water and reprecipitated by half-saturation with $(NH_4)_2SO_4$. The filtration of the ammonium sulphate solutions was always slow.

The impure precipitate of depocaseose was dissolved in water and $Ba(OH)_2$ solution was added with thorough stirring until the solution was distinctly alkaline. The precipitate of $BaSO_4$ was filtered off and thoroughly washed with hot water. The combined filtrate and washings were evaporated *in vacuo* to remove ammonia and the excess Ba ions removed quantitatively with H_2SO_4. The filtrate and washings were concentrated *in vacuo* to a thick syrup (about

200 ml.) and poured with thorough stirring into at least 10 vol. of absolute alcohol. The depocaseose, thus thrown out in granular form, was ground up and dried with successive changes of absolute alcohol. A further quantity was obtained by evaporating the alcoholic liquors, pouring into absolute alcohol and drying as above. The combined crops were finally dried *in vacuo* over H_2SO_4 and consisted of a fine almost white powder. The yields of material were:

	Batch I	Batch II
	g.	g.
Casein taken	480	640
Depocasein	170 = 35%	280 = 44%
Depocaseose	90 = 19%	100 = 16%

The rather low yields of depocaseose are due to loss in removing $(NH_4)_2SO_4$ and incomplete precipitation by alcohol.

The analyses of these preparations compared with the casein, all dried at 105°, were:

	N	P	S	Ash
	%	%	%	%
Casein	15·08	0·82	0·63	1·10
Depocasein batch I	13·84	0·03	0·45	0·49
Depocasein batch II	13·78	0·03	0·42	0·35
Cf. Plimmer & Lawton [1939]	13·50	0·03	0·43	0·70
Depocaseose batch I	14·46	0·04	0·69	0·81
Depocaseose batch II	14·57	0·02	0·67	0·98

Depocaseose has the same low P content as depocasein, but slightly higher N and S contents. Both depocasein and depocaseose have lower N contents than caseinogen owing to removal of amide-N by the action of alkali.

Determination of arginine, histidine and lysine

The basic amino-acids in depocasein and depocaseose and casein for comparison were determined by the micro-method of Block [1934] with the modifications proposed by Plimmer & Lowndes [1938] and by Block [1938] using 2·5 g. protein. The main alteration was in the use of silver sulphate added as Ag_2O and H_2SO_4 to avoid loss of lysine. The introduction of excess Ag ion in this way was not altogether satisfactory as the operation was lengthy and a positive test for excess Ag ion was not always obtainable. On this account the results for histidine may be low. The use of limited amounts of $AgNO_3$ [Tristram, 1939] is probably preferable. These analyses were completed before the publication of his thorough and critical review of the method. The finding by Ayre [1938] that ammonium phosphotungstate is not decomposed by dilute H_2SO_4 in the presence of amyl alcohol-ether, rendering the removal of NH_3 with NaOH unnecessary, was not followed. The isolation of arginine flavianate always gave between 70 and 80% of the yield expected from the N value of the solution, though the salt crystallized readily from the hot solution. Histidine diflavianate was also too low in amount partly on account of the small quantity present and partly from solubility loss in washing free from excess flavianic acid. The sulphur contents of both arginine and histidine flavianates were higher than the theoretical values, which agrees with the observations of Tristram and others. The use of nitranilic acid for isolation of histidine as suggested by Block and proved quantitative [1940] should be an improvement of the method. The isolation of lysine picrate proved the most difficult, probably owing to the formation of some more soluble dipicrate. In agreement with the findings of other workers we found that lysine picrate gives a decomposition point with slight explosion only after recrystallization. A new indicator "Sofnol Purple" which changes from green to blue at

*p*H 7·4 and from blue to purple at *p*H 9 was used in later analyses. As it does not contain N it can be added to the solutions if required. Solubility corrections of the salts were not applied to the results. They have been studied very fully by Tristram with simple mixtures of the three amino-acids, but their application to protein hydrolysates should be accepted with reserve. The data of our analyses were:

Amino-acid: % of dry protein

	Arginine		Histidine		Lysine	
	By N	From flavianate	By N	From flavianate	By N	From picrate
Depocaseose	3·53	2·34	0·73	—	8·65	4·91
	3·02	2·01	0·99	0·90	7·33	5·30
	3·44	2·70	—	—	7·44	—
	2·94	2·22	0·88	0·76	(9·45)	6·10
Mean	3·23	—	0·87	—	7·81	—
Depocasein	4·87	3·23	0·90	0·71	5·76	2·34
	4·31	3·17	—	—	5·43	4·00
	4·66	2·56	1·05	0·62	5·86	—
Mean	4·61	—	0·98	—	5·70	—
Cf. Plimmer & Lawton	—	3·32	—	1·58	—	4·49
Casein	3·3	2·1	1·33	1·05	7·0	6·2
Cf. Vickery & White [1933]	—	3·85	—	1·83	—	6·25

The figures for depocasein differ considerably from those of Plimmer & Lawton, who used the macro-method of Vickery & Leavenworth [1926–8]. The differences are explicable in part by the actual *p*H taken in precipitating histidine silver, the shade of the blue-green of the bromophenol blue being easily capable of considerable variation in interpretation by different eyes. Lysine picrate in small quantities is difficult to precipitate quantitatively. We regard the figures by N determination as more accurate than those by isolation.

On the present figures depocaseose contains less arginine and more lysine than casein, whereas depocasein has more arginine and less lysine. Histidine is less in both than in the original protein.

Expressed in terms of N as % of total N the results are:

% of total N

	Arginine-N	Histidine-N	Lysine-N
Depocaseose	7·2	1·62	10·6
Depocasein	10·7	1·95	8·2
Cf. Plimmer & Lawton	7·9	3·1	6·5
Casein	7·0	2·4	9·1
Cf. Vickery & White	8·2	3·2	7·7

The molecular ratios of the amino-acids calculated by dividing the percentage figures by the mol. wt. and taking the nearest probable whole number are:

	Histidine	Arginine	Lysine	Histidine	Arginine	Lysine
Depocaseose	0·0056	0·0185	0·0535	1	3	9
Depocasein	0·0063	0·0265	0·0390	1	4	6
Cf. Plimmer & Lawton	0·0102	0·0191	0·0310	1	2	3
Casein	0·0086	0·0190	0·0480	1	2	5
Cf. Vickery & White	0·0117	0·0221	0·0430	1	2	4

The ratios for casein agree nearly with those from Vickery & White's analyses. For depocasein the ratios from our analyses agree with those by Plimmer & Lawton for arginine and lysine, but not for histidine.

Determination of cystine and methionine

These amino-acids were determined by the method of Baernstein [1936] with the alteration of Plimmer & Lowndes [1937]. The alterations suggested by Kassell & Brand [1938] were considered unnecessary as their results were the same in the case of casein as those of Plimmer & Lowndes. The results of our analyses of casein and depocaseose compared with those of depocasein and cow's caseinogen were:

		Casein %	Depocaseose %	Depocasein %	Cow's caseinogen %
Cystine		0·27	0·23	—	—
		0·31	0·25	—	—
	Mean	0·29	0·24	0·30	0·36
Methionine		2·59	2·84	—	—
		2·54	2·74	—	—
	Mean	2·57	2·80	2·09	2·89
S as H_2S etc.		0·04	0·14	—	—
		0·05	0·13	—	—
	Mean	0·04	0·13	—	—

If the percentage of S in the protein be calculated from these data (cystine, 26·7 % S; methionine, 21·5 % S) the total S is slightly higher than that determined directly by Pirie's method [1932]:

	Casein	Depocaseose	Depocasein	Cow's caseinogen
Cystine-S	0·077	0·064	0·080	0·096
Methionine-S	0·553	0·602	0·449	0·621
H_2S-S	0·040	0·130	—	—
Total	0·670	0·796	0·529	0·717
Total S direct	0·63	0·69	0·45	0·65

The small differences are probably within the range of experimental error magnified by the calculations involved. They do not justify the small factor corrections of Kassell & Brand which would increase the differences still further.

Expressed in terms of amino-acid-N as percentage of total N the data are:

	Casein	Depocaseose	Depocasein	Cow's caseinogen
Cystine-N	0·224	0·193	0·24	0·28
Methionine-N	1·60	1·81	1·35	1·78

On calculating the molecular ratios of $\frac{\text{methionine}}{\text{cystine}}$ the differences in the proteins are striking:

Casein	Depocaseose	Depocasein	Cow's caseinogen
14·3	18·8	11·2	12·9

For every molecule of cystine there are 14 molecules of methionine in casein, 13 in cow's caseinogen, 19 in depocaseose and 11 in depocasein.

Determination of tyrosine and tryptophan

The combination of the colorimetric methods of Folin & Looney [1922] and Folin & Ciocalteu [1927] as recommended by Plimmer & Lawton [1939] was used to determine tyrosine and tryptophan. Tryptophan was also determined by the bromination method of Plimmer & Lowndes [1938]. The determinations were

almost completed before the appearance of Lugg's [1938] method of hydrolysis with alkaline stannite. The results of these determinations were:

	Tyrosine			Tryptophan		
	By Millon reagent %	By phenol reagent %	Mean %	By bromination %	By phenol* reagent %	Mean %
Casein	5·95	5·16	—	1·44	1·24	—
	5·74	—	5·62	—	—	1·34
Depocasein	8·28	7·75	—	1·21	1·12	—
Batch II	8·33	—	8·12	—	—	1·17
Cf. Plimmer & Lawton	8·15	7·20	7·68	1·39	1·24	1·31
Depocaseose	3·01	3·67	—	1·01	0·92	—
Batch I	3·07	3·21	3·24	—	—	0·97
Batch II	3·41	3·75	3·57	0·99	0·84	0·92

* Mean of determinations by HgS and NaCN methods.

The Millon reagent gave slightly higher results than the phenol reagent for casein and depocasein, but the reverse was the case with depocaseose. As previously observed the bromination method for tryptophan gives higher results than the phenol reagent, probably owing to precipitation of traces of histidine. It is noticeable that the higher tyrosine content of depocasein than of casein is compensated by the lower content in depocaseose. Tryptophan is slightly lower in depocaseose.

The data are very similar if the results be expressed in terms of tyrosine-N:

N as % of total protein-N

	Casein	Depocasein	Depocaseose (batch II)
Tyrosine-N	2·88	4·55	1·90
Tryptophan-N	1·22	1·20	0·90

Determination of phenylalanine

The colorimetric method of Kapeller-Adler [1932] and its modification by Block [1938], in which phenylalanine is nitrated to 3:4-dinitrobenzoic acid and the reduction of this compound to di-aci-dihydro-dinitrobenzoic acid which has a deep purple colour, was not successful with our solutions of protein hydrolysates. Dark brown or dark-coloured solutions were always obtained which were impossible to match in a Duboscq colorimeter with standard phenylalanine solutions which gave a typical purple colour. The method is evidently satisfactory with solutions containing fewer amino-acids obtained during fractionation of the amino-acids as in the experiments by Baptist & Robson [1940]. Arnaud [1939] does not give details of the modification by which he determined the phenylalanine content of hen's egg albumin.

Determination of glycine

Patten's colorimetric method [1935] for estimation of glycine with *o*-phthalic dialdehyde, which had proved unsatisfactory in the trials by Plimmer & Lawton was not again attempted. Town's semi-micro-method [1936] of precipitating with nitranilic acid, reported as specific in the absence of NH_4, Na, K, Ca and Ba ions, was used.

Numerous experiments were made in which pale yellow precipitates, not always crystalline, presumably of glycine nitranilate, slowly settled out. After

washing with alcohol, drying *in vacuo* over H_2SO_4 and weighing, their purity was examined by dissolving in water (in several experiments a portion remained insoluble) and estimating the nitranilic acid by precipitation with $BaCl_2$ and the N (glycine) in the filtrate. The results indicated that the precipitate was a mixture. The method tested on pure glycine, using 11–20 mg., gave a recovery of only 64–80 %. With larger amounts of glycine such as are contained in gelatin and other proteins the recovery would be greater. Our figures gave 2·1 and 2·5 % of glycine in casein and 2·1–2·6 % in depocaseose, values which are considerably higher than those of 0·5 % [Patten, 1935] and 0·45 % [Foreman, 1919] and 1 % [Plimmer & Lawton, 1906] for casein. With the small quantities of glycine nitranilate obtained from casein and depocaseose it was not possible to make 3:5-dinitrobenzoyl derivatives as was done with gelatin hydrolysates by Town, who obtained a crude yield of 82 % and a yield of 27 % of pure substance. Town gave no proof that *only* glycine was precipitated by nitranilic acid.

Our investigations were resumed later after Block [1940] had shown that histidine nitranilate was quantitatively precipitated. Our high figures would thus be explained. It may also be noted that histidine nitranilate contains 59·8 % nitranilic acid compared with 60·5 % in glycine nitranilate, values so close that the nitranilic acid content of the precipitate does not indicate whether glycine or histidine or both are present.

New estimations were carried out in which NH_4 ions and basic amino-acids were removed by precipitation with phosphotungstic acid. 2 g. protein were hydrolysed by boiling with 40 ml. of 20 % HCl for 24 hr. The solution was evaporated several times *in vacuo* to remove excess HCl. The syrup was dissolved in water, heated nearly to boiling and about 20 ml. of 40 % phosphotungstic acid in *N*/2 HCl were added with stirring till precipitation was complete. Next day the precipitate was filtered off and washed with *N*/2 HCl. The combined filtrate and washings were shaken twice with 20 ml. amyl alcohol-ether mixture to remove excess phosphotungstic acid and the clear aqueous solution evaporated *in vacuo* and brought to 25 ml. 10 ml. portions were evaporated to smaller bulk and transferred to centrifuge tubes so that the volume was 3–4 ml., and absolute alcohol added to make 80 %. Any precipitate which formed was filtered off and 30 mg. or known excess of nitranilic acid dissolved in a few ml. absolute alcohol were added. The pale yellow precipitate of glycine nitranilate slowly formed and settled out. It was centrifuged, washed and analysed for nitranilic acid as barium salt. The results were:

	Glycine nitranilate		Glycine (mg.)		% glycine in protein		
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	Mean
Casein 1·788 g.	10·0	7·0	2·5	2·0	0·35	0·28	0·32
Depocasein 1·80 g.	3·6	6·1	1·2	2·0	0·17	0·27	0·22
Depocaseose 1·80 g.	8·0	5·2	2·0	1·3	0·28	0·19	0·24

The value for glycine was calculated from the Ba nitranilate precipitate; in each case the nitrogen of the filtrate (glycine) from barium nitranilate corresponded.

The values thus obtained are lower than the accepted value for casein. As shown above the recovery of glycine with such small quantities is between 60 and 80 %. The true values of glycine in the three proteins are probably between 0·5 and 0·6 %. More satisfactory results would be obtained by using larger quantities of protein.

Determination of glutamic acid by enzyme micro-method

Cohen [1939] introduced a method of determining glutamic acid consisting in its oxidation with chloramine-T to cyanopropionic acid which is then hydrolysed to succinic acid and extracted from the solution with ether. The succinic acid is dissolved in phosphate buffer and estimated by determination of oxygen uptake under the action of succinic dehydrogenase in a Warburg apparatus. The method is specific for glutamic acid and its derivatives, glutamine and glutathione. The adaptation of the method to the estimation of glutamic acid in protein hydrolysates was not actually tried by Cohen. Dr Cohen was interested in this development of his method, kindly carried out an analysis and gave a demonstration.

Before carrying out estimations of glutamic acid in protein hydrolysates it seemed advisable to make experiments with glutamic acid, under the conditions used in the actual analyses:

Glutamic acid			
Taken mg.	Found mg.	% recovery	Time of ether extraction
20	13·0	65	3 hr.
12	8·9	75	5 ,,
16	12·4	78	5 ,,
20	18·0	90	5 ,,
16	13·3	83	5 ,,
16	12·6	80	5 ,,
20	15·9	80	Overnight
24	20·6	85	Overnight

The recovery was much less than the 95 % recorded by Cohen, who regarded the loss as largely due to over-oxidation. In these experiments a larger quantity of chloramine-T was used: 4 ml. of 20 % solution as compared with 2 ml. of 10 % by Cohen. The higher quantity here taken was necessary to allow for oxidation of the other amino-acids in the protein hydrolysates. According to Dakin [1917] the molar concentration of chloramine-T should be at least twice that of glutamic acid. In the case of the hydrolysates the loss due to over-oxidation would be much reduced or eliminated as may be noticed from the comparative results from isolation (below).

10 g. of each protein were hydrolysed by boiling with 20 % HCl for 24 hr. The HCl was partially removed from the solutions by evaporation *in vacuo* and completely by treatment with Ag_2O and H_2SO_4. Ag was removed with H_2S and the latter by evaporation *in vacuo*. The acid solutions were made up to 250 ml. and aliquots taken for N estimation and acidity.

Volumes (4–5 ml.) of the solutions (=150–200 mg. protein) containing 20–25 mg. glutamic acid were placed in small conical flasks, brought to *p*H 4–5 with approx. 0·5 ml. dil. NaOH and 3 ml. citrate buffer added which brought the *p*H to 4·7; 4 ml. of 20 % chloramine-T solution were added drop by drop with thorough shaking (a precipitate of *p*-toluenesulphonamide appeared) and the flasks placed in a rack and shaken at 40° for 10 min. The flasks were then placed in an ice bath for 20 min. so as to precipitate most of the *p*-toluenesulphonamide or excess reagent. The solutions were filtered and the precipitates washed with several small volumes of water and the combined filtrate and washings, 25–30 ml., collected in large boiling tubes. Conc. HCl was added to give a concentration of not less than 12·5 % and the tubes placed in boiling water for 15 min. to hydrolyse the cyanopropionic acid to succinic acid. After allowing to cool 40 % NaOH was added dropwise until the solutions became hot again, at which point

1 ml. of 5% NH_4Cl was added to decompose traces of chloramine-T. Several drops of phenol red were then added and the solutions made alkaline to a purple colour, avoiding a large excess of alkali. The cool solutions were transferred to a continuous ether extractor with small quantities of water used to wash the boiling tubes and extracted with freshly distilled peroxide-free ether for 2 hr. to remove traces of *p*-toluenesulphonamide. The solutions were then acidified strongly to phenol red with 20% H_2SO_4 (usually 3–4 ml.) and extracted for 4–5 hr. into a clean extraction flask during which time the phenol red and succinic acid were removed. 2–3 ml. *M*/10 phosphate buffer were added to the ether solutions in the flasks, followed by dropwise addition of 2*N* NaOH until the indicator showed neutrality or slight alkalinity (red-purple). The ether was distilled off and the aqueous solutions concentrated on a steam bath to 1–2 ml. and transferred to small graduated cylinders. The extraction flasks were rinsed two or three times with 1 ml. *M*/10 phosphate, the washings added to the solutions in the cylinders and the total volume of each solution recorded. They were kept within 5 ml., as it was necessary to have approximately 4 mg. succinic acid per ml. for satisfactory readings in the subsequent manometric estimation. The apparatus used for this purpose was the Warburg type of constant volume with simple reaction flasks with which Keilin cups were employed. 4 ml. suspension from pigeon breast muscle, prepared as described by Cohen, were placed in the main compartment and the volume of succinic acid taken was between 0·2 and 0·5 ml., depending on the capacity of the Keilin cup. The oxygen uptake was measured.[1] The amount of glutamic acid in the hydrolysate was calculated from

$$\mu l.\ O_2 \times \frac{1{\cdot}47}{112} = \text{mg. glutamic acid}$$

or 1 mg. glutamic acid = 76·2 μl. O_2 uptake. The results were:

Hydrolysate	Glutamic acid in 4 ml. mg.		% in protein	Cf. by isolation
Casein 3·56 %	(23·4)		(16·6)	—
	31·2		21·6	—
	32·6		22·9	—
		Mean	22·3	21·0 [Dakin, 1918]
By Cohen	—		20·6	—
Depocasein 3·70 %	34·7		23·6	—
	32·9		22·0	—
	35·7		24·1	—
		Mean	23·3	3·6 (Plimmer & Lawton)
By Cohen	—		15·4	—
Depocaseose 3·75 %	28·6		19·1	—
	(25·4)		(16·8)	—
	28·9		19·5	—
		Mean	19·2	17·5 (see below)
By Cohen	—		19·9	—

The figure for casein corresponds well with the figure by isolation; it is higher than the isolated figure in the case of depocaseose. The isolated figure for depocasein is undoubtedly too low. The general agreement of the figures shows the value of the enzyme method of Cohen for estimating glutamic acid. Depocasein has a slightly greater amount of glutamic acid than casein. As might be expected depocaseose has a relatively smaller quantity. In terms of glutamic acid N as % of total protein N the figures are: casein 14·1; depocasein 16·2; depocaseose 12·6.

[1] These experiments were carried out in the Biochemistry Dept., Oxford. We desire to express our thanks to Prof. Peters for allowing the use of his laboratory.

Determination of hydroxyamino-acids

A. *Threonine.* Block & Bolling [1939] introduced a micro-method of determining threonine consisting in its oxidation with lead tetra-acetate to acetaldehyde, which is aerated into concentrated H_2SO_4 and condensed with *p*-hydroxydiphenyl to give a red-violet colour.

In preliminary experiments to test the method certain difficulties were encountered and some modifications had to be introduced. It was advantageous to insert an extra tube or Drechsel wash-bottle containing purified acetic acid between the spray tube (2) and the reaction tube (3). By this means the volume of solution in tube (3) was kept approximately constant, thus correcting for acid carried over into tubes (4) and (5). Tube (5) containing pellets of solid NaOH often became clogged with sodium acetate which stopped the air current and held back traces of acetaldehyde. It was replaced by a second empty tube placed in ice water with tube (4). Tube (2*a*) or Drechsel bottle and the reaction tube (3) were kept covered with black paper to stop effects believed to be due to sunlight on lead tetra-acetate and acetic acid. In sunlight it was found that with only the reagents present a blue colour developed in the absorption tube (6) different in colour from the red-violet produced by acetaldehyde. Even with purified acetic acid a blank aeration of at least 1 hr. was necessary to remove traces of acetaldehyde from reagents. With threonine solution (standard or unknown) the aeration had to be continued for 2–3 hr.; 1 hr. was insufficient with 1 mg. threonine. The colour comparison was made with a Duboscq type of colorimeter with a blue filter (Ilford) to cut out red and yellow shades as recommended by Block & Bolling.

In practice, a blank aeration was carried out for 1 hr. and if no colour developed in tube (6) a known quantity of standard threonine solution (0·5–1 mg.) was placed in reaction tube (3) containing 1 g. $Pb(Ac)_4$ in purified glacial acetic acid and air drawn through at a steady slow rate for about 2·5 hr. during which time the colour attained maximum density. Tube (6) was then disconnected, placed in boiling water for 2 min. to remove excess of *p*-hydroxydiphenyl and the clear solution after cooling in ice was kept at room temperature. A fresh absorption tube (6) was attached and aeration continued to ensure that all acetaldehyde had been removed. The same procedure was repeated with the unknown solution.

0·5–1 ml. of a 0·1 % solution of threonine synthesized by the method of West & Carter [1937] was used as standard.

The protein solutions were prepared by boiling 1–2 g. protein with 10–20 ml. 20 % HCl for 24 hr., evaporating several times *in vacuo* and treating with Ag_2O suspension to remove Cl ions. Ag was removed with H_2S and the filtered solution evaporated to dryness on a water bath. After standing *in vacuo* over H_2SO_4 the residue was dissolved in purified glacial acetic acid and brought to 50 ml. Total N was determined in an aliquot; 2 ml. gave colours comparable with 1 mg. threonine. The results were:

		% threonine in dry protein	Threonine-N % of total N
Casein		2·8	—
		3·7	—
	Mean	3·3	2·58
Depocasein		1·92	1·64
Depocaseose		1·80	—
		2·10	—
	Mean	1·95	1·60

B. *Total β-hydroxy-α-amino-acids.* Van Slyke *et al.* [1940] pointed out that β-hydroxy-α-amino-acids were oxidized by potassium periodate in alkaline solution with quantitative liberation of their N as NH_3. This reaction was studied by Nicolet & Shinn [1939]. Investigations by Mr Lowndes in this department with threonine, serine and hydroxyglutamic acid gave results fully agreeing with Van Slyke's statement.

The same hydrolysates were used as for the threonine estimations above. The operation is carried out in three aeration tubes as commonly employed for NH_3 estimations. First, 10 ml. hydrolysate were made strongly alkaline to phenolphthalein with K_2CO_3 and NH_3 from amide groups aerated into standard acid. Powdered KIO_4 was then added to the tube and NH_3 aerated into a fresh tube with standard acid for 1 hr. The results were:

	NH_3 from amide groups as N	NH_3 from β-hydroxy-amino-acids as N	% of total protein-N	
			Amide-N	β-Hydroxy-amino-acid-N
Casein 10 ml. hydrolsate = 22·4 mg. N	2·41	1·80	10·75	8·05
Depocasein 10 ml. hydrolysate = 40·9 mg. N	2·92	2·06	7·20	5·05
Depocaseose 10 ml. hydrolysate = 54·3 mg. N	2·82	3·68	5·20	6·80

The amide-N for casein corresponds with the usual figure of 10–12 %. The figures for depocasein and depocaseose are lower on account of liberation of NH_3 during the alkaline digestion of casein. The figures for hydroxyamino-acids in both depocasein and depocaseose are lower than in casein. Unless alkaline digestion of casein produces some special action on these hydroxyamino-acids the greater amount in depocaseose suggests that depopeptone will contain still higher amounts, which corresponds to their order of solubility in water.

Deducting threonine-N from the total hydroxyamino-N, the difference represents serine-N and hydroxyglutamic acid-N:

In casein, $8·05 - 2·58 = 5·47$.
In depocasein $5·05 - 1·64 = 3·41$.
In depocaseose $6·80 - 1·60 = 5·20$.

Our estimations of serine (below) were not successful. The amount of serine in casein determined by isolation is quoted as $0·5 = 0·44$ % serine-N of total N. This would leave 5·0 % N as hydroxyglutamic acid-N = 8·7 % amino-acid. Dakin's figure was 10·5 %. In contrast Rapoport [1935] gave 8·1 % of glycine and serine in casein calculated as glycine. Assuming 0·5 % glycine calculation shows that there would then be little or no hydroxyglutamic acid in casein. Harington & Randall [1931] failed to isolate this amino-acid and Gulland & Morris [1934] only obtained 0·33 %.

C. *Serine.* Rapoport's method [1937] of estimating serine, consisting in deamination with nitrous acid to glyceric acid and condensation with naphthoresorcinol in conc. H_2SO_4 to give a blue colour, has been tried with the hydrolysates of these proteins. In the final stage a dry residue containing the glyceric acid is suspended in 2 ml. conc. H_2SO_4 containing 0·1 % reagent and heated on the water bath for 1 hr. In all cases a brown charred solution was obtained without any blue colour. We are unable to account for the lack of success with this method.

Isolation of monoamino and dicarboxylic acids in depocaseose

The amino-acids other than those estimated by special methods were determined as far as possible by isolation.

50 g. depocaseose were hydrolysed by boiling with 1500 ml. 20 % HCl for 24 hr. and the solution evaporated *in vacuo* several times to remove excess HCl. The solution of the resulting syrup, filtered from humin material, was brought to a known volume for determination of total N. This figure corresponded to 42·2 g. depocaseose in 1 litre used for isolation.

Removal of basic amino-acids. A test portion of 50 ml. was found to be precipitated by 22 ml. phospho-24-tungstic acid [Wu, 1920] solution. The remainder of the solution was heated and precipitated with 418 ml. The actual quantity of phosphotungstic acid required was 4·16 g. per 1 g. depocaseose. After several days at 0° the precipitate of phosphotungstates was filtered off and washed three times with $N/10$ HCl. The clear filtrate and washings were combined and shaken with about 200 ml. of amyl alcohol-ether mixture to remove excess phosphotungstic acid and evaporated to about 30 ml.

Precipitation of glutamic acid HCl. The concentrated solution was saturated with HCl gas and kept for several days at 0°. The crystalline precipitate G_1 was filtered off, washed with fuming HCl and dried *in vacuo* over NaOH and CaO. The filtrate was evaporated to much smaller volume and again saturated with HCl gas. Two further precipitates G_2 and G_3 were obtained and dried *in vacuo* over NaOH and CaO.

G_1 weighed 0·4 g., contained 1 % N and consisted mainly of inorganic material.

G_2 and G_3 together weighed 14·2 g. and contained 8·6 % N and 22·4 % Cl. On recrystallization from sat. HCl two crops G_a of 7·8 g. with 7·74 % N and 19·9 % Cl and G_b of 0·31 g. with 7·5 % N and 18·8 % Cl were obtained (glutamic acid HCl has 7·63 % N and 19·3 % Cl). The yield corresponds to 6·6 g. glutamic acid.

The filtrates from the recrystallized crops of glutamic acid HCl were evaporated *in vacuo* to remove HCl. Cl ions were removed with sat. Ag_2SO_4 solution, Ag with H_2S, and after evaporation *in vacuo*, SO_4 and NH_4 ions with $Ba(OH)_2$ solution and finally Ba ions quantitatively with H_2SO_4. On evaporating to a small bulk two crops of glutamic acid (calc. N = 9·5 %) mixed with leucine (calc. N = 10·69 %) crystallized out, G_c of 2·84 g. with 10·16 % N and G_d of 0·4 g. with 10·26 % N. During recrystallization some of the solution was lost, but 0·8 g. glutamic acid with N = 9·3 % and 0·7 g. leucine with N = 10·27 % were isolated. From one of the mother liquors a precipitate of 0·06 g. with N = 8·0 % and M.P. 230°, possibly phenylalanine (calc. N = 8·4 %), was obtained. The yields were thus 7·4 g. glutamic acid, 0·7 g. leucine, 0·06 g. phenylalanine.

Precipitation of proline and hydroxyproline as reineckates. The main filtrate of amino-acids from the crops of glutamic acid HCl was evaporated *in vacuo* several times. The residual syrup was dissolved in 100 ml. water and treated with 200 ml. 10 % aqueous solution of ammonium reineckate [Kapfhammer & Eck, 1927; Bergmann, 1935]. After 2 days at 0°, the reddish brown precipitate R_1 was filtered off. On concentration of the filtrate and addition of more reineckate two further crops R_2 and R_3 were obtained on standing for 2 days at 0°. The combined precipitates weighed 4 g. and were suspended in about 100 ml. water and treated with 10–20 ml. pyridine. The dark oily residue which settled out was filtered off and washed with difficulty. The solution was made alkaline with $Ba(OH)_2$ solution and evaporated *in vacuo* to remove NH_3 and pyridine. A small

insoluble residue was filtered off and Ba ions removed quantitatively with H_2SO_4. The filtrate and washings were acidified with HCl and a solution of ammonium rhodanilate in MeOH added. No crystalline precipitate was obtained, nor could any hydroxyproline or proline be isolated from the solution. Only dark gummy residues settled out.

The dark coloured solution from the reineckate precipitate containing HCl and pyridine was heated and treated with hot saturated Ag_2SO_4 solution until precipitation was complete. Most of the colour was removed. The clear filtrate was evaporated *in vacuo* to 200 ml., Ag removed with H_2S and the solution again evaporated. It was made alkaline with NH_4OH and evaporated *in vacuo* to remove pyridine and excess NH_3. The almost neutral solution, about 75 ml., was kept *in vacuo* over H_2SO_4 for 7 days. A crystalline precipitate of impure tyrosine T_1 slowly formed. The filtrate and washings on further evaporation gave a second crop T_2. These crops, weighing 1·1 g. with 9·0 % N and 0·5 g. with 10·9 % N, were recrystallized from very dilute NH_4OH, followed by evaporation and addition of small quantities of alcohol. Four crops of crystals resulted: (1) 0·45 g. with 7·6 % N, almost pure tyrosine (calc. N = 7·73 %); (2) 0·28 g. with 9·15 % N, a mixture of equal parts of tyrosine and leucine; (3) 0·3 g. with 10·15 % N, mainly leucine (calc. N = 10·69 %) and (4) 0·31 g. with 13·3 % N, possibly valine (calc. N = 12·97 %). The yields of tyrosine and leucine would be 0·6 g. and 0·45 g.

Separation of calcium aspartate. The filtrate from crop T_2 was treated with hot $Ba(OH)_2$ solution to remove SO_4 ions and the alkaline filtrate evaporated *in vacuo* to remove NH_3. Excess Ba ions were removed quantitatively with H_2SO_4 and the filtrate and washings, about 100 ml., were made strongly alkaline with a 10 % suspension of $Ca(OH)_2$ and filtered from excess. The remaining colour was thus removed from the solution which was evaporated to a small volume and to which 10 vol. of absolute alcohol were slowly added with careful stirring. A white precipitate of Ca salts settled out and was filtered off after 24 hr. The precipitate weighing 4·5 g. was dissolved in warm water, filtered from a trace of insoluble matter and treated with the exact amount of oxalic acid to remove Ca ions. After evaporation to a small volume it was saturated with HCl gas. The small precipitate consisted of inorganic matter. HCl was removed by evaporation and with Ag_2SO_4, Ag with H_2S, SO_4 ions with $Ba(OH)_2$ and the latter quantitatively with H_2SO_4 and the clear neutral solution was boiled with excess $CuCO_3$. The dark blue solution on evaporation to 25 ml. gave crystals of Cu aspartate, which were filtered off, washed with cold water and air-dried. The yield was 1·28 g. The crystals lost 0·959 g. at 100° *in vacuo* over P_2O_5 = 24 % (Cu aspartate + 3·5 H_2O = 24·4 % H_2O). The anhydrous salt contained 32·1 % Cu (calc. Cu = 32·5 %). The yield corresponds to 0·67 g. aspartic acid. Addition of small quantities of alcohol failed to produce further crystallization. The solution was freed from Cu with H_2S and evaporated to 10 ml. No crystalline precipitate formed and alcohol produced a gummy residue. The alcoholic solution evaporated *in vacuo* gave a very deliquescent residue on standing over H_2SO_4, which weighed 0·67 g. and contained 10·9 % N and some Cl. Cl was removed and ultimately 0·4 g. of white crystals with 11·8 % N was obtained. This was believed to be proline (calc. N = 12·1 %). The residue insoluble in alcohol was dissolved in water and the solution poured into alcohol. A powder, slightly coloured, weighing 0·8 g. was obtained. A white residue, insoluble in water, weighing 0·21 g., was obtained from it. Its N content was 7·4 % and it gave Millon's reaction and was tyrosine. From the insoluble Ca salts 0·67 g. aspartic acid, 0·21 g. tyrosine and 0·4 g. proline (?) were thus obtained.

The alcoholic filtrate from the insoluble Ca salts was evaporated, filtered from $CaCO_3$ and the Ca removed quantitatively with oxalic acid. On further evaporation crystalline plates appeared which were redissolved on heating the solution and came out again on cooling M_1. The filtrate and washings on evaporation gave a series of further crops M_2–M_7 and more crops M_8–M_{13} were obtained by adding small quantities of alcohol. The final solution contained only 0·17 g. N from which no more crystals could be obtained. The yields and N contents of the crops were:

M_1	0·33 g.	with 10·67 % N	Correspond to 2·9 g. leucine (calc. N = 10·69 %)
M_2	0·65	10·40	
M_3	0·20	10·22	
M_4	0·54	10·54	
M_5	0·26	10·70	
M_6	0·56	10·90	
M_7	0·36	10·45	
M_8	0·50	11·2	Corresponds to 0·3 g. leucine + 0·2 g. valine.
M_9	0·33	11·9	Correspond to 1·8 g. valine (calc. N = 11·96 %) and traces of alanine or lower amino-acids.
M_{10}	0·25	12·65	
M_{11}	0·76	11·9	
M_{12}	0·15	11·8	
M_{13}	0·31	11·4	

The total yields of amino-acids isolated and with their N expressed in terms of total depocaseose-N were:

	Isolated g.	% of depocaseose	Amino-acid-N	% of total depocaseose-N
Glutamic acid	7·4	17·5	0·703	11·5
Tyrosine	0·81	1·9	0·063	1·03
Leucine	4·35	10·3	0·466	7·6
Valine	2·0	4·9	0·24	4·0
Aspartic acid	0·67	1·6	0·07	1·14
Proline?	0·4	1·0	0·048	0·8
Unidentified N	—	—	0·17	3·5
			0·042	—

The quantity of glutamic acid is nearly the same as that determined by the enzyme method, but the amount of tyrosine is much lower than that by colorimetric determination. The values for leucine are probably near the actual amounts in depocaseose, but the amount of aspartic acid is probably too low.

The optical activity of isolated amino-acids

In view of Dakin & Dudley's observations [1912; 1913] on the racemization of the amino-acids produced by the action of $N/2$ NaOH at 37° for 18–20 days on casein it was of interest to examine the rotations of the amino-acids obtained from depocaseose. The crops of leucine and valine were recrystallized in two main fractions and used for these experiments.

Glutamic acid. 5·35 % in 9 % HCl in 2 dm. tube gave $\alpha = 2{\cdot}68°$, $[\alpha]_D = +25{\cdot}0°$; pure *l*-acid in 9 % HCl = +31·0°.

Leucines. 3·45 % in 20 % HCl in 2 dm. tube gave $\alpha = 1{\cdot}80°$, $[\alpha]_D + = 26{\cdot}1°$; *l*-leucine in 20 % HCl = +15·9°; *l-iso*leucine in 20 % HCl = +37·3°.

Valine. 2·72 % in 20 % HCl in 2 dm. tube gave $\alpha = 1{\cdot}02°$, $[\alpha]_D = +18{\cdot}5°$; *l*-valine in 20 % HCl = +28·8°.

Both valine and glutamic acid have thus undergone a small amount of racemization by the action of $N/4$ NaOH at 37° for 24 hr. No conclusion can be drawn from the leucine specimen as it is probably a mixture of the isomers.

Summary and Discussion

The compositions of caseinogen, depocasein and depocaseose are compared in the following tables:

g. of amino-acids as % of dry protein

	Caseinogen	Depocasein	Depocaseose
Cystine	0·29	0·30*	0·24
Methionine	2·57	2·09*	2·80
Tyrosine	5·62	8·12	3·57
Tryptophan	1·34	1·17	0·94
Arginine	3·30	4·61	3·23
Histidine	1·33	0·98	0·87
Lysine	7·00	5·70	7·81
Glutamic acid	22·30	23·30	19·20
Threonine	3·30	1·92	1·95
β-Hydroxyamino-acids other than threonine	7·65	4·0	6·8
Glycine	0·32	0·22	0·24
Ammonia	1·94	1·20	0·91
Aspartic acid	4·1*	3·0*	1·6
Leucines	9·4*	11·5*	10·3
Valine	7·9*	0·3*	4·9
	77·36	68·41	65·36

The figures marked * under caseinogen are from Dakin's results and those under depocasein from Plimmer & Lawton's.

The figure for β-hydroxyamino-acids is an approximate estimate based on the N content (next table) assuming an average molecular weight of these amino-acids (serine and hydroxyglutamic acid) = 130.

The last three amino-acids have not been estimated by the same methods. No importance can be attached to the figures from a comparative point of view.

Amino-acid-N as % of total N

	Caseinogen	Depocasein	Depocaseose
Cystine	0·22	0·25	0·19
Methionine	1·60	1·42	1·81
Tyrosine	2·88	4·55	1·90
Tryptophan	1·32	1·20	0·90
Arginine	7·00	10·70	7·20
Histidine	2·40	1·95	1·62
Lysine	9·10	8·20	10·60
Glutamic acid	14·10	16·20	12·61
Threonine	2·58	1·64	1·60
β-Hydroxyamino-acids other than threonine	5·47	3·41	5·20
Glycine	0·40	0·31	0·31
Ammonia	10·75	7·20	5·20
Aspartic acid	2·85	2·30	1·14
Leucines	6·90	9·30	7·60
Valine	6·30	0·20	4·00
	72·87	68·83	62·08

There is great similarity in the compositions of caseinogen, depocasein and depocaseose. A marked difference only appears in tyrosine and threonine and other β-hydroxyamino-acids. Small differences are observable in methionine, lysine and glutamic acid. They may however be significant as they may indicate an unequal distribution of the amino-acids between depocasein and depocaseose. The differences are shown more clearly in the molecular ratios obtained by

dividing the % amount of the amino-acid by its molecular weight and then taking cystine as unity, or from the N figures making allowance for the number of N atoms in each molecule.

Ratio of amino-acids from g. mol. per 100 g. protein

	Caseinogen	Depocasein	Depocaseose
Cystine	1	1	1
Methionine	14	11	19
Tyrosine	26	36	20
Tryptophan	6	5	5
Arginine	16	21	19
Histidine	8	5	6
Lysine	40	32	54
Glutamic acid	126	127	132
Threonine	24	13	16
Other β-hydroxyamino-acids	50	26	54
Glycine	4	3	4
Ammonia	100	56	54
Aspartic acid	26	18	12
Phosphorus	22 atoms		

The principle of Bergmann & Niemann [1936] to calculate from the analytical figures the frequencies of the amino-acids from the formula:

$$F = \frac{100 \times W}{G \times A} \text{ where } W = \text{mol. wt. of the amino-acid,}$$
$$G = \% \text{ wt. of the amino-acid in the protein,}$$
$$A = \text{average mol. wt. of all amino-acids,}$$

requires the assumption of the mean mol. wt. A. The value of F depends on the value assigned to A and on the accuracy of the value of G. Small changes in these figures affect F considerably. When applied to depocaseose the frequencies could not be expressed in powers of 2 or 3. As an absolute value it has little significance as a characteristic of the protein. The simple ratios in the above table show the variations in the compositions of the three proteins more clearly.

Certain of the amino-acids now appear to be distributed unequally between the two products from caseinogen:

Methionine and lysine show marked differences; there is a greater amount in depocaseose and less in depocasein than in casein.

β-Hydroxyamino-acids including threonine are more abundant in depocaseose than in depocasein. It is possible that the larger number of OH groups which are hydrophilic accounts for the greater solubility of the proteose.

The tyrosine content of the products may also be of significance with regard to solubility. Tyrosine is present in greatest amount in depocasein. All the tyrosine of casein is accounted for in the two fractions, depocasein and depocaseose, and thus the still more soluble peptone should not contain this amino-acid (see below). The 24 hr. alkaline digestion of casein splits off almost exactly half of the amide groups. The NH_3 from proteins is generally assumed to be present as amide groups on the dicarboxylic acids. The extent to which free carboxyl groups are combined in casein appears to be $\frac{100}{126+26}$ (assuming the aspartic figure to be correct) = 65 %. Damodaran [1931] found it to be 55 %.

The other amino-acids, tryptophan, arginine, histidine, glutamic acid etc. occur in almost equal proportions in depocasein and depocaseose.

Molecular weights of the proteins. Assuming that each molecule of protein contains one molecule of cystine the molecular weights calculated from the g. mol. of cystine per 100 g. protein are:

casein $\frac{100}{0{\cdot}0012}=83{,}300$: depocasein $\frac{100}{0{\cdot}00125}=80{,}000$: depocaseose $\frac{100}{0{\cdot}0010}=100{,}000$.

The view of Sörensen [1930] that caseinogen is a reversibly dissociable system of closely similar components in a state of equilibrium depending on the conditions of the solution, reached also by Svedberg & Carpenter [1930] by ultracentrifugal methods (the components having mol. wt. 75,000–100,000), is strengthened by these analyses. The action of dilute NaOH produces a change in the complex system with the formation of two main components depocasein and depocaseose.

The action of 1 % NaOH on casein may be summarized as follows:

(*a*) Hydrolysis of the ester linkages by which the phosphoric acid is believed to be bound.

(*b*) Hydrolysis of approximately half the dicarboxylic acid amide groups during 24 hr.

(*c*) Some change as a result of which the complex system of casein is separated into two main components, depocasein and depocaseose, which may or may not be homogeneous. These products from chemical data appear to be of the same molecular size between 75,000 and 100,000, and they exhibit small yet significant differences in amino-acid composition.

It is believed that the differences in properties, such as solubility between the metaprotein and proteose are dependent on the amino-acid composition and not on molecular size.

(*d*) It appears probable from mol. wt. and other data that dilute NaOH does not attack peptide linkages to any great extent if at all. It may be noted that Dakin & Dudley found that after separation of racemized casein and caseose only 1/10 of the N remained in solution, i.e. after 20 days the peptide chains have not been broken more than after 1 day.

Composition of depopeptone. A speculative view based on the above conception of the casein molecule would be that the peptone consisted of molecules of the same size as depocasein and depocaseose, but having a greater proportion of hydrophilic groups (hydroxyamino-acids) to account for its greater solubility. Estimations of the amino-acids in the peptone can be calculated from the figures of the analyses of the three proteins. There should be no tyrosine and hydroxy-amino-acid-N should be high. A preliminary experiment with 1·3 g. impure peptone containing 14·1 % N showed the absence of tyrosine, but the hydroxy-amino-acid-N was only 3·7 % of the total N. A special series of experiments will be needed to prepare sufficient of the peptone to make full analyses.

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172. A COMPARATIVE STUDY OF ARGINASE AND CANAVANASE

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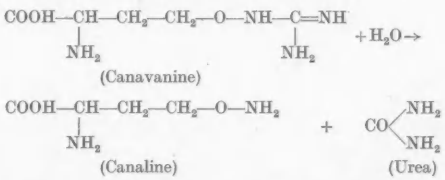
(*Received 9 August 1940*)

Very little is known about the enzyme canavanase which brings about the decomposition of canavanine into canaline and urea:



The existence of the enzyme was assumed by Kitagawa & Tomita [1929] because it was found that in the presence of liver tissue or extract canavanine gave rise to urea according to the above equation. It was the object of this investigation to study the reaction in more detail and especially to compare it with the hydrolysis of the closely similar amino-acid arginine by arginase as there are several grounds for suspecting the possible identity of the two enzymes. Canavanine has not been shown to be a constituent of proteins, in spite of the fact that the properties of this amino-acid are such that it should readily be identifiable in protein hydrolysates. It appears doubtful if the organism would contain an enzyme for dealing with a substance which it is not likely to encounter in metabolism. Further, modern studies on enzyme specificity have amply demonstrated that susceptibility to enzymic decomposition of any substance is determined not by the empirical composition but by the chemical groupings present in it.

The experiments to be described concerned the following questions: kinetics of the hydrolysis of arginine and canavanine by liver preparations and by jack bean meal which latter has been recently shown to contain arginase [Hellerman & Perkins, 1935–6; Stock *et al.* 1938]; the influence of activators and inhibitors; comparison of arginase and canavanase activities of preparations obtained by different methods, and the relative distribution of the two enzymes in different sources.

In kinetics the two enzymes showed only such differences as could be explained by the difference in the substrates. Using dehydrated preparations obtained by precipitating aqueous liver extract with acetone, the optimum *p*H for the splitting of arginine was found to be about 9·4 (Fig. 3) and for canavanine about 7·5 (Fig. 3), the range of enzymic activity in the former case being *p*H 5·5–11·5 and in the latter 5·5–10·5. This however provides no evidence for the existence of two separate enzymes as it has been shown by Felix & Schnieder [1938] that while arginase acts on arginine at a *p*H-optimum of 9·5 the *p*H-optima for its action on other substrates, viz. argininic acid, methylaminoarginine and

arginylarginine lie between 7 and 8. When the effect of *p*H was examined in the presence of cobalt ions (shown by Hellerman & Perkins [1935–6] to activate arginase) it was found that the *p*H-optima for the hydrolysis of both arginine and canavanine coincide at about 7·5 (Fig. 4). Further, the enzyme of jack bean meal also showed approximately the same optimal *p*H (Fig. 6) in its action on these two substrates in the presence of cobalt chloride. For both the reactions it was found that the initial reaction velocity was approximately proportional to the enzyme concentration (Tables 1 and 2). From the *p*S-activity curves the optimal substrate concentration for arginine is seen to be $M/15$ and for canavanine $M/3$, the values for the Michaelis constant for the two reactions being $0{\cdot}0118\,M$ and $0{\cdot}0371\,M$ respectively (Fig. 5).

The evidence from the preparative experiments is wholly in support of the identity of the two enzymes. Not only were all attempts to separate the two enzymes unsuccessful but in no case was it possible to obtain a preparation which catalysed the hydrolysis of canavanine at a higher rate than the hydrolysis of arginine. The kinetic experiments show that under optimal conditions for each enzyme the initial velocity of hydrolysis of canavanine is about 1/6th of the velocity of hydrolysis of arginine. On account of experimental difficulties it was not possible to determine the velocities of the two reactions with different preparations under strictly comparable conditions, i.e. under optimal conditions for each enzyme. But it was always found that whatever the procedure adopted for preparation, the effect on canavanase and arginase was always in the same direction and approximately to the same extent. Thus autolysis of liver tissue as a preliminary to precipitation of the enzyme tended to decrease the activities of both the enzymes (Table 3) and in preparations obtained after various periods of autolysis, the ratio of the amount of arginine hydrolysed to canavanine hydrolysed remained constant (Tables 3, 4 and 5). Autolysis of liver at different *p*H gave similar results. Autolysis at *p*H 7 yielded a more active preparation than at the natural *p*H of the liver (6·2), but here again the relative velocities of the two reactions were unaffected. Autolysis at *p*H 9 for 24 hr. destroyed both the enzymes.

The distribution of the enzymes in liver, kidney and blood of various animals and in certain seeds showed the same general tendency. Livers of rat, guinea-pig, rabbit and ox are rich in arginase and canavanase while kidneys of the above animals contain moderate amounts of both. Both the enzymes are absent from the blood of rat, guinea-pig and rabbit and both are present in the blood of ox and cow.

Jack bean meal recently shown by Stock *et al.* [1938] to contain arginase was also found to act on canavanine. As the presence of arginase in urease preparations used for urea determinations is a matter of practical importance a number of sources of urease recently studied in this laboratory [Damodaran & Sivaramakrishnan, 1937] were examined for arginase and canavanase activities. Seeds of *Cucurbita maxima* (common gourd), *Citrullus vulgaris* (water melon), *Dolichos biflorus* (horse-gram) and *Soja hispida* (soya bean) showed no appreciable action on either arginine or canavanine, while the seeds of *Canavalia obtusifolia* and *C. ensiformis* (jack bean) acted vigorously on both, the action on canavanine again being slower than that on arginine.

Activation and inhibition experiments also lead to the same conclusion that the two enzymes are identical. Cobaltous ions activate and cyanide inhibits both reactions; and further in the case of liver the range of enzymic activity in the presence of cobalt for both enzymes is shifted by about one *p*H unit towards the acid side. With the enzymes from both the sources after activation with

cobalt the maximum activity for the hydrolysis of arginine and canavanine is found to be near *p*H 7·7. The balance of evidence appears thus to be in favour of the identity of the two enzymes.

Experimental

Substrates. *l*(+)-Arginine monohydrochloride was prepared from gelatin by the method of Cox [1928] and canavanine from the seeds of *Canavalia obtusifolia* according to Damodaran & Narayanan [1939]. Analytically pure specimens of both were used.

Enzymes. A dehydrated liver preparation was obtained according to Edlbacher [1917] with slight modifications. Ox liver, which was brought in ice from the slaughter-house, was cut into small pieces, washed free from blood with cold water and passed through a mincer twice. The minced tissue (500 g.) was ground for half an hour in a mortar with acid-washed sand and distilled water (1 ml. per g. of the moist tissue) and after adjusting to *p*H 7, by the addition of a few ml. of *N*/20 NaOH, kept in the refrigerator overnight under toluene. The mixture was centrifuged, and after removing toluene the filtrate poured into 10–15 vol. of acetone with stirring. The supernatant liquid was decanted, the precipitate filtered at the pump, washed with further quantities of acetone, dried first in a current of air and then in a vacuum desiccator over H_2SO_4. The dry material was ground finely and sieved through muslin. Such a preparation was very active both on arginine and canavanine and retained its activity unimpaired for long periods.

From the dry enzyme preparation the enzyme solution for each experiment was prepared as follows: weighed amounts of the powder were ground up in a glass mortar to a thin paste with water, brought to *p*H 7, and the mixture made up to a known volume with water. After 2 hr. at 37° under toluene it was filtered and the filtrate mixed with an equal volume of glycerol. The solution, kept in the refrigerator under toluene, retained its activity for more than a month.

Enzyme action was in general determined as follows. The neutralized substrate solution was mixed with the buffer of appropriate *p*H (McIlvaine's phosphate-citric, Sørensen's phosphate and Sørensen's glycine buffers were used for the ranges of *p*H 3–5·8, 5·8–8·0 and 8·0–12·5 respectively) and after addition of enzyme solution was made up to known volume. Toluene was used as antiseptic. In aliquots withdrawn at intervals enzyme action was arrested by the addition of 1 ml. of *N* H_2SO_4 and keeping in a water bath at 80° for 10 min. Urea was determined according to Conway & Byrne [1933] using water-melon urease [Damodaran & Sivaramakrishnan, 1937]. Two controls were run, (1) enzyme solution incubated without the substrate, and (ii) substrate solution incubated with boiled enzyme.

*The p*H *activity curves of arginase and canavanase*

Reaction mixture. 10 ml. substrate solution, *M*/10 (urea-N on complete hydrolysis 28·02 mg.) + 10 ml. of *M*/5 buffer + 10 ml. of enzyme solution. Total volume 50 ml.

In the case of arginine an enzyme solution obtained by extracting 0·5 g. of the liver powder with 50 ml. of water was used and in that of canavanine one obtained by extracting 2 g. of the powder with 50 ml. of water. Figs. 1 and 2 give the activities of canavanase and arginase at various *p*H plotted against time. From these graphs the time taken to hydrolyse 50 % of the substrate at each *p*H was obtained. In Fig. 3 the reciprocal of the half-value period is plotted

against *p*H for each enzyme. As seen from Fig. 3 the optimum *p*H for canavanase is 7·5 and for arginase 9·4.

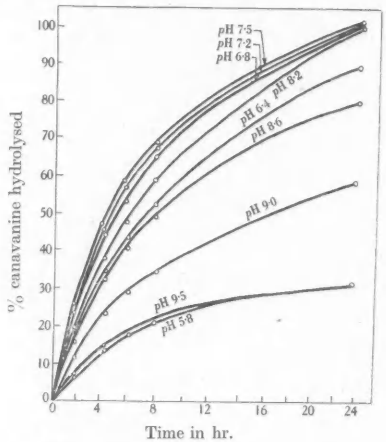


Fig. 1.

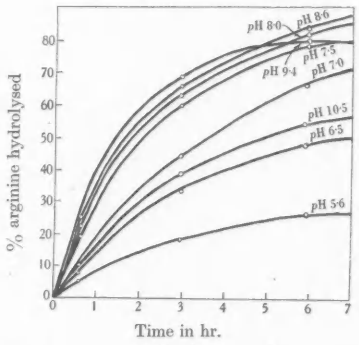


Fig. 2.

Fig. 1. Hydrolysis of canavanine by liver enzyme at various *p*H.

Fig. 2. Hydrolysis of arginine by liver enzyme of various *p*H.

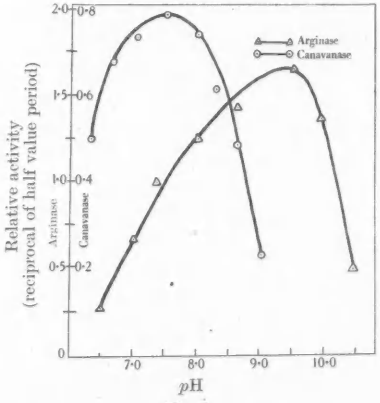


Fig. 3.

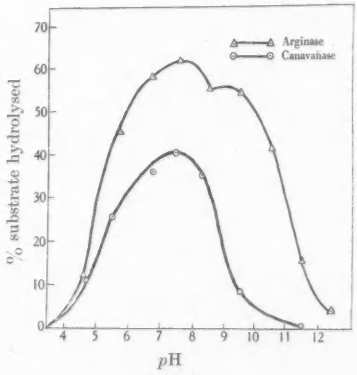


Fig. 4.

Fig. 3. Activity-*p*H curves of arginase and canavanase.

Fig. 4. Activity-*p*H curves for liver arginase and canavanase in the presence of cobalt chloride.

Activity-pH curves of liver arginase and canavanase in the presence of cobalt chloride

Reaction mixture. 5 ml. of substrate solution ($M/10$) + 5 ml. of buffer + 2 ml. of cobalt chloride solution (6 mg.) + 5 ml. of enzyme solution (0·5 g. of the liver powder extracted with 50 ml. of water). Total volume 25 ml. The activity-*p*H curves for the two enzymes are given in Fig. 4.

Hydrolysis of arginine and canavanine by jack bean seed meal at different p*H*

Procedure. To 2 ml. of the substrate solution ($M/5$) in a 50 ml. conical flask, 5 ml. of required buffer (M), 2 ml. of distilled water and 5 ml. of a suspension of jack bean meal (0·1 g.) were added. The flask was fitted with a one-holed rubber stopper closed by a glass tap. After incubation for 3 hr. at 37°, 2 ml. of N H_2SO_4 were added through the tap tube, the enzyme destroyed by keeping the flask in a boiling water bath for 10 min. and the solution filtered into a 25 ml. measuring flask. Urea, the product of the reaction mixture, does not accumulate owing to the simultaneous presence of urease in the reaction mixture. Control experiments with equivalent quantity of urea solution at *p*H 9·0, 9·5 and 10·5 showed

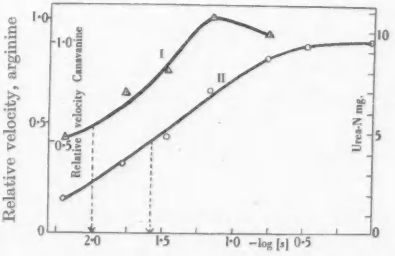


Fig. 5. Activity-*p*S curves. I, arginase *p*H 9·4; II, canavanase *p*H 7·5.

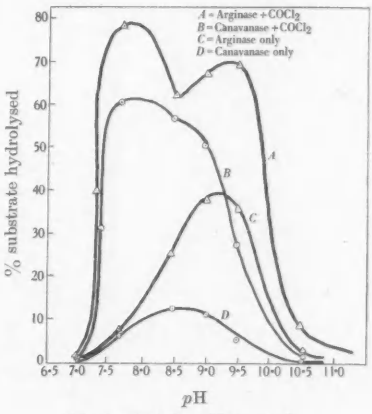


Fig. 6. Activity-*p*H curves for jack bean enzyme.

that the urea was completely hydrolysed at *p*H 9·0 in 3 hr., while at *p*H 9·5 90 % was hydrolysed and at *p*H 10·5 no hydrolysis occurred. In experiments at *p*H $<9{\cdot}0$ the filtrate in the measuring flask was made up to the mark and ammonia determined on an aliquot. In experiments at *p*H $>9{\cdot}0$ the filtrate in the measuring flask was brought to *p*H 7 and further digested for 30 min. with 0·5 ml. of 10 % suspension of water-melon urease, the solution made up to the mark and ammonia determined on an aliquot. Control experiments with water-melon urease showed that it does not contain either arginase or canavanase.

The activity-*p*H curves for jack bean arginase and canavanase in the absence and in the presence of cobalt chloride are given in Fig. 6. The hydrolysis of arginine and canavanine by jack bean meal is accelerated by the presence of cobalt chloride and the amount of activation depends upon the *p*H. That the enzyme from jack bean meal behaves in a slightly different way from that of ox liver is evident from the activity-*p*H curves for these enzymes shown in Fig. 6. Neither arginine or canavanine is hydrolysed by jack bean meal at *p*H 6·6 while at the same *p*H liver enzyme is noticeably active towards both substrates. The optimum *p*H for jack bean arginase and canavanase in the presence of cobalt chloride is about 7·7 and in this respect the enzyme resembles that from ox liver in the presence of cobalt chloride.

The influence of enzyme concentration

Reaction mixture. 10 ml. of substrate solution ($M/10$)+5 ml. of buffer ($M/5$ phosphate, pH 7·5 for canavanine and $M/5$ glycine pH 9·4 for arginine)+x ml. of enzyme solution (0·5 g. of the liver powder extracted with 50 ml. of water). Total volume 25 ml. The results are given in Tables 1 and 2 and the reaction velocity curves for the two substrates are given in Figs. 7 and 8.

Table 1. *Proportionality between enzyme concentration and urea formation from canavanine*

Enzyme quantity ml. E	Time in hr. T	ET	Urea-N formed in mg.
1	6	6	2·103
2	3	6	2·151
4	1½	6	2·101
2	2	4	1·601
4	1	4	1·552

Table 2. *Proportionality between enzyme concentration and urea formation from arginine*

Enzyme quantity ml. E	Time in min. T	ET	Urea-N formed in mg.
0·5	100	50	3·651
1	50	50	3·602
2	25	50	3·600
1	100	100	5·701
2	50	100	5·652
4	25	100	5·653

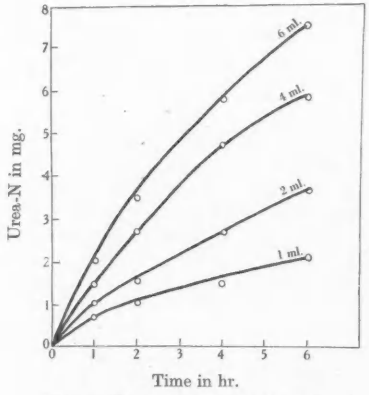


Fig. 7. Enzyme concentration and hydrolysis of canavanine.

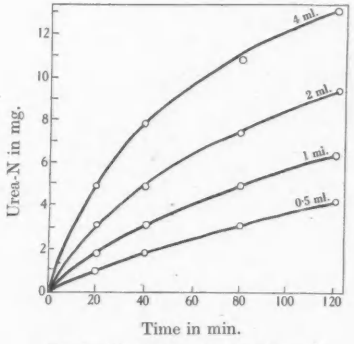


Fig. 8. Enzyme concentration and hydrolysis of arginine.

The influence of substrate concentration

Reaction mixture. 20 ml. of substrate solution, 20 ml. of buffer ($M/10$ phosphate pH 7·5 for canavanine and $M/10$ glycine pH 9·4 for arginine), 20 ml. of enzyme solution (1 g. of the liver powder extracted with 50 ml. of water).

As shown in Figs. 9 and 10 both the reactions depend upon the substrate concentration. In the case of arginine the maximum amount of urea formed is reached at a final concentration of $M/15$, while in the case of canavanine the maximum is reached only at a final concentration of $M/3$; further, in the case of arginine on increasing the concentration to $M/6$ a slight inhibition is observed.

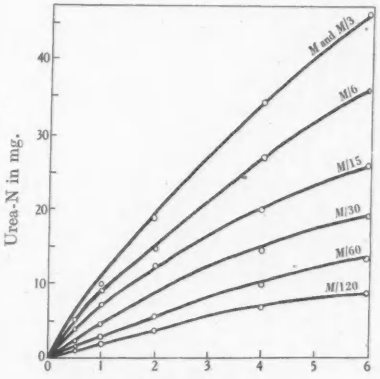


Fig. 9. Substrate concentration and hydrolysis of canavanine at *p*H 7·5.

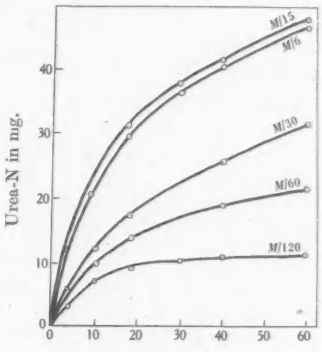


Fig. 10. Substrate concentration and hydrolysis of arginine at *p*H 9·4.

Effect of autolysis on the extractability of arginase and canavanase

Fresh ox liver was extracted with water as described in the preparation of the enzyme and the mixture kept at room temperature (30°) for various periods. After 16, 40 and 64 hr. portions of the mixture were centrifuged and the filtrate dehydrated with acetone in the usual way. The arginase and canavanase activities of the three preparations thus obtained were then tested. The results are given in Table 3.

Table 3. *Effect of autolysis for different periods on arginase and canavanase activity*

	mg. of urea-N formed after 4 hr. incubation		
Enzyme	Arginine	Canavanine	Urea-N from arginine / Urea-N from canavanine
16 hr. filtrate	9·85	7·59	1·30
40 hr. filtrate	9·02	6·91	1·30
64 hr. filtrate	9·02	6·21	1·47

Reaction mixture. 5 ml. of substrate solution (urea-N on complete hydrolysis 10 mg.) + 5 ml. buffer (*p*H 7·5 for canavanine and *p*H 9·4 for arginine) + 5 ml. of enzyme solution (1 g. in 100 ml. of water).

*Effect of autolysis at different p*H *on arginase and canavanase activity*

Portions of ox liver extract were kept at room temperature at the *p*H of the liver (6·2), at *p*H 7·0 and at *p*H 9·0. After 24 hr. the mixtures were centrifuged and the filtrate dehydrated with acetone. The arginase and canavanase

activities of the three preparations thus obtained were then determined, the reaction mixture being the same as in the previous experiments. The results are given in Table 4.

Table 4. *Effect of autolysis at different* p*H on arginase and canavanase activities*

Enzyme	mg. of urea-N formed after 4 hr. incubation		
	Arginine	Canavanine	Urea-N from arginine / Urea-N from canavanine
*p*H 6·2 filtrate	8·54	3·83	2·23
*p*H 7·0 filtrate	9·06	4·03	2·24
*p*H 9·0 filtrate	0	0	—

Fractionation of liver extract with sodium sulphate

The precipitates obtained by treatment of liver extract with sodium sulphate to half and full saturation were tested for arginase and canavanase activities. The precipitates were suspended in water equal to the original volume of the extract and 5 ml. of the suspension were used as the enzyme solution. The reaction mixture was the same as in the previous experiment and the results are given in Table 5.

Table 5. *Effect of precipitation of liver extract with* Na_2SO_4 *on arginase and canavanase activities*

Enzyme	mg. of urea-N formed after 4 hr. incubation		
	Arginine	Canavanine	Urea-N from arginine / Urea-N from canavanine
Precipitate obtained after half saturation with Na_2SO_4	9·88	3·31	2·98
Precipitate obtained after full saturation with Na_2SO_4	9·60	2·85	3·36
Filtrate from the above	1·96	0·55	3·51

Distribution of arginase and canavanase in the tissues of different animals (Table 6)

The animals tested were all adults. The guinea-pigs, rats and rabbits were killed in the laboratory. The tissues and blood of ox and cow were brought in ice from the slaughter-house.

Table 6. *Distribution of arginase and canavanase in the tissues and blood of different animals*

Source	mg. of urea-N formed after 4 hr. incubation					
	Liver		Kidney		Blood	
	Arg.	Can.	Arg.	Can.	Arg.	Can.
Guinea-pig (male)	9·381	8·412	2·113	0·671	Nil	Nil
Guinea-pig (female)	9·546	7·193	2·451	1·452	,,	,,
White rat (male)	9·123	8·470	3·976	1·061	,,	,,
White rat (female)	9·123	8·021	3·976	1·112	,,	,,
Rabbit (male)	9·548	8·470	1·184	0·293	,,	,,
Rabbit (female)	9·123	7·941	1·412	0·285	,,	,,
Ox	9·581	8·573	0·513	0·354	7·521*	1·925*
Cow	9·541	7·791	0·812	0·354	5·491*	2·110*

* 18 hr. incubation instead of 4 hr.

Enzyme solution. The fresh tissue (5 g.) was extracted with water (25 ml.), the mixture centrifuged and the supernatant liquid made up to 50 ml. so that 1 ml. of the solution corresponded to 0·1 g. of the moist tissue. The blood was diluted with an equal volume of water.

Reaction mixture. 5 ml. of substrate solution (urea-N 10 mg.)+5 ml. of buffer (*p*H 7·5 for canavanine and *p*H 9·4 for arginine)+5 ml. of enzyme solution. Total volume 25 ml.

Distribution of arginase and canavanase in different urease preparations (Table 7)

The different sources of urease tested were the seeds of *Citrullus vulgaris* (water melon), *Canavalia obtusifolia*, *Soja hispida* (soya bean), *Dolichos biflorus* (horse-gram), *Cucurbita maxima* (common gourd) and *Canavalia ensiformis* (jack bean). In all cases the ground material was used without removal of fat. In the case of water melon seeds, which were tested, the results obtained were the same as those obtained with undefatted material.

Table 7. *Hydrolysis of arginine and canavanine by urease preparations from different sources*

	mg. of urea-N formed after 3 hr. incubation			
	Arginine		Canavanine	
Source	mg.	%	mg.	%
Citrullus vulgaris (water melon)	0·072	1·3	0·072	1·3
Soja hispida (soya bean)	0·110	2·0	0·097	1·7
Cucurbita maxima (common gourd)	0·063	1·1	0·051	0·9
Dolichos biflorus (horse-gram)	0·100	1·8	0·068	1·2
Canavalia obtusifolia	3·880	69·4	2·970	53·0
C. ensiformis (jack bean)	4·460	79·6	3·452	61·7

Among the different sources of urease examined only the seeds of *Canavalia obtusifolia* and jack bean contain an enzyme which under suitable conditions hydrolyses both arginine and canavanine. Water melon urease does not possess this property.

Reaction mixture. 2 ml. of substrate solution (*M*/5)+5 ml. of *M* buffer *p*H 7·7+2 ml. of cobalt chloride solution (6 mg.)+5 ml. of an aqueous suspension of urease (0·1 g.).

Comparison of arginase and canavanase activities of liver enzyme in the absence and in the presence of $CoCl_2$

(I) Without addition of cobalt chloride.

Reaction mixture. 5 ml. of substrate solution (*M*/10)+5 ml. of buffer of *p*H 7·5+5 ml. of enzyme solution (0·5 g. of the liver powder extracted with 50 ml. of water). Total volume 25 ml. The results are given in Table 8.

Table 8. *Arginase and canavanase activities in absence of $CoCl_2$*

	Urea-N				
	Arginine		Canavanine		
Time hr.	mg.	%	mg.	%	Urea-N from arginine / Urea-N from canavanine
1	3·246	23·2	0·875	6·3	3·71
2	5·615	40·1	1·486	10·6	3·78
3	7·190	51·4	2·002	14·3	3·61
4	8·598	61·4	2·436	17·4	3·58

(II) In the presence of cobalt chloride.

The reaction mixture was the same as in the previous experiment but 2 ml. of cobalt chloride (6 mg.) solution were added before the addition of the enzyme. The results are given in Table 9.

Table 9. *Arginase and canavanase activities in presence of cobalt chloride*

Time hr.	Urea-N: Arginine mg.	Urea-N: Arginine %	Urea-N: Canavanine mg.	Urea-N: Canavanine %	Urea-N from arginine / Urea-N from canavanine
1	5·615	40·1	3·414	24·4	1·64
2	8·706	62·1	5·698	40·7	1·52
3	11·420	81·6	7·491	53·5	1·52
4	13·451	96·1	9·143	65·3	1·47

Effect of cyanide

The actions of liver enzyme on arginine and canavanine at their respective *p*H-optima were studied with and without the addition of potassium cyanide.

The results are shown in Figs. 11 and 12 from which the inhibitory influence of cyanide at high concentrations is evident. Under given conditions canavanase

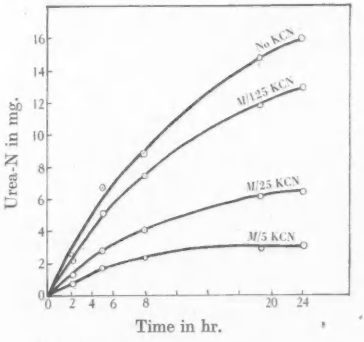


Fig. 11. Effect of KCN on the hydrolysis of canavanine.

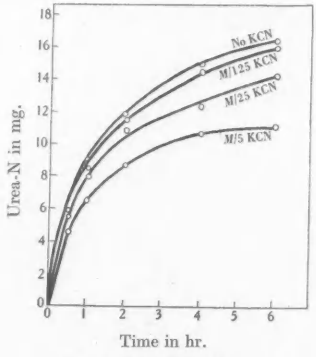


Fig. 12. Effect of KCN on the hydrolysis of arginine.

is inhibited to a greater extent than arginase. Such a preferential inhibition is probably to be ascribed to the difference in *p*H as shown by Edlbacher *et al.* [1932].

Reaction mixture. 10 ml. of substrate solution ($M/10$) + 10 ml. of buffer + 10 ml. of neutralized KCN solution + 10 ml. of enzyme solution (1 g. of the powder extracted with 50 ml. of water). Total volume 50 ml.

Summary

A comparative study of the enzymes arginase and canavanase from both animal and plant sources has been made.

In their kinetics the two enzymes show only such differences (*p*H-optimum, optimum substrate concentration and Michaelis constant) as are to be expected from the nature of the substrates.

In other respects there is a close parallelism between arginase and canavanase actions. In tissues and blood of different animals they are either present together or not at all and in every case the action on canavanine is slower than that on arginine. By no procedure has it been possible to reverse this quantitative relationship and obtain a preparation in which canavanase activity is stronger than arginase activity. In any particular preparation factors affecting arginase influence canavanase in the same direction and often to the same extent, e.g. the loss of activity on autolysis and the increase in activity in the presence of cobalt ions affect both enzymes equally. In the presence of cobalt the *p*H-optima for the two reactions also become identical at *p*H 7·5.

The balance of evidence is thus in favour of the two enzymes being identical.

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173. ESTIMATION OF OXYHAEMOGLOBIN AND OF METHAEMOGLOBIN BY A PHOTOELECTRIC METHOD

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(*Received 9 August 1940*)

The oxyhaemoglobin content of blood can be rapidly and accurately estimated with the help of a photoelectric colorimeter. In the absence of oxyhaemoglobin or of other pigments, methaemoglobin can be determined in the same way. In whole blood the presence of met- or of sulph-haemoglobin or of other pigments can be detected. The experiments also indicate that the use of 0·4 % ammonia as a diluent for blood for spectrophotometric measurements is not safe. Better results can be obtained with a concentration of 0·04 % ammonia.

Instrument

The photoelectric colorimeter used for these experiments was a Hilger "Absorptiometer" [Hilger, 1936]. The light source of this is a projector lamp from the white light of which suitable spectral bands can be selected with the help of filters. Two beams of light fall on two photocells which are connected in opposition across a galvanometer so that, when the photoelectric currents given by the cells are equal, the galvanometer shows zero deflexion. The instrument works with a compensation-null method which ensures a high accuracy. The accuracy of the absorptiometer used in the present experiments was better than 0·3 %.

The optical densities of the solutions to be measured are read off directly from the instrument scale. The optical density is the logarithm of the ratio of the intensity of the incident light to the intensity of the transmitted light. This function has the advantage that it is approximately linear with the concentration of the absorbing substance. However, since the light source of the absorptiometer is not monochromatic, the density is not strictly proportional to the concentration and therefore calibration curves are necessary.

Estimation of oxyhaemoglobin

To prepare a calibration curve for oxyhaemoglobin, whole blood of known haemoglobin concentration was diluted with 0·04 % ammonia[1] in various proportions and the optical densities of the diluted solutions were read with a green or a blue filter.[2] In the present experiments the resulting calibration curve with the green filter was actually a straight line over the range of haemoglobin concentrations from 0·5 to 1·1 mg./ml. (see curve 1, Fig. 1). Calibration curves prepared with normal blood samples of different origin showed a variation up to 1 %. This means that if two bloods give the same density reading for example at a dilution of 1 in 100, they may differ as much as 1 % at a dilution of for instance 1 in 300.

[1] 0·04 ml. of ammonia (sp.gr. 0·880) made up to 100 ml. with water.

[2] Chance Bros. Colour filters, Nos. 5 and 6 respectively.

The actual estimation of oxyhaemoglobin was carried out as follows. The blood specimen to be examined, which contained sodium citrate or potassium oxalate as anticoagulant, was diluted with 0·04 % ammonia to a concentration giving a density reading of 0·25–0·5, one part in two hundred usually being a suitable ratio, and the oxyhaemoglobin concentration was read off directly from the calibration curve. The time required for the whole estimation was only

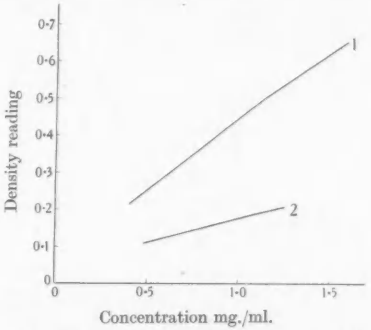

Fig. 1. Calibration curves for oxyhaemoglobin. Curve 1: green filter; 1 cm. cell. Curve 2: "Spectrum Orange" filter; 4 cm. cell.

2–3 min., and the relative accuracy was limited only by the 1 % uncertainty of the calibration curve explained in the previous paragraph. The absolute accuracy depends naturally on the method by which the haemoglobin content of the calibration solutions has been determined. In the present experiments this has been done with a Hilger spectrophotometer.

Estimation of methaemoglobin

In the absence of oxyhaemoglobin or of other pigments, methaemoglobin can be estimated in a similar way. The solutions, both for the calibration and for the experiments described below, were prepared by diluting one part of blood, containing anticoagulant, with four parts of water, together with a few drops of a freshly prepared concentrated solution of potassium ferricyanide. The absorption of blue light by the ferricyanide prohibits the use of blue or violet filters, but at these concentrations its effect is negligible with green, yellow or red filters. The concentrations of methaemoglobin in the calibration solutions were calculated from the haemoglobin concentration of the original blood.

Detection of other absorbing substances

If, using monochromatic light, calibration curves for oxyhaemoglobin are prepared at two different wave-lengths, the optical densities at these wave-lengths for a solution which contains only oxyhaemoglobin should correspond to the same concentration; but if another pigment, e.g. met- or sulph-haemoglobin, or haematin, is also present, they will not do so, and the presence of this foreign pigment can be detected. A marked effect would be expected with these substances since they absorb at long wave-lengths much more intensely than does haemoglobin itself [cf. Heilmeyer, 1933].

Using the absorptiometer, this principle was applied by constructing calibration curves for pure oxyhaemoglobin solutions (*a*) with the Chance's green filter

mentioned above and a 1 cm. cell; and (*b*) with an Ilford "Spectrum Orange" filter,[1] which has a narrow range of transmission at about 5900 Å, with a 4 cm. cell (see Fig. 1). In this way, suitable density readings for both filters were obtainable with one solution. The blood to be tested was diluted with 0·04 % ammonia to a suitable concentration and the apparent concentration was determined with either filter. If these two apparent concentrations were not the same, a foreign pigment was present.

As an example, it was found that in a solution containing 0·8 mg. haemoglobin per ml. the presence of 3 % of methaemoglobin increased the reading with the "Spectrum Orange" filter by 10 %, while that with the green filter was unchanged.

Normal blood samples of different origin differed from each other up to about 5 % in the corresponding density readings, i.e. solutions of different normal bloods, giving the same reading with the green filter, gave up to 5 % different density readings with the "Spectrum Orange" filter. Such variations have been observed by other workers using spectrophotometric methods [cf. Heilmeyer, 1933]. This 5 % uncertainty would correspond in the case of the example cited above to 1·5 % uncertainty in the detection of methaemoglobin. This means that the presence of more than 1·5 % methaemoglobin can be detected with certainty, and also very small concentrations of other foreign pigments. The method will not, however, detect carboxyhaemoglobin, in view of the similarity of its absorption to that of oxyhaemoglobin; nor can it be used for very turbid blood, since light scattering then affects the density readings.

The use of 0·4 % ammonia as diluent

Readings of the type described above revealed that in 0·4 % ammonia the conversion of oxyhaemoglobin into methaemoglobin, and of methaemoglobin into haematin proceeds much faster than is usually supposed, and considerable changes were found to occur within 1 hr. after the blood was diluted. The changes are most marked during estimations involving methaemoglobin and could lead to serious errors in spectrophotometric measurements.

Table 1 shows the conversion of oxyhaemoglobin into methaemoglobin by ammonia at room temperature. It contains successive readings on different samples of the same blood, using the "Spectrum Orange" filter. 1 % increase in

Table 1. *Conversion of oxyhaemoglobin into methaemoglobin in presence of ammonia*

("Spectrum Orange" filter)

Time after dilution		20 min.	80 min.	4 hr. 20 min.	18 hr.	43 hr.
0·4 % ammonia: Sample	1	0·163	0·171	0·184	0·228	0·281
„	2	0·162	0·171	0·185	0·228	0·282
„	3	0·163	0·170	0·185	0·227	0·282
0·04 % ammonia: „	4	0·163	0·165	0·167	0·173	0·182
„	5	0·162	0·164	0·167	0·169	0·180
„	6	0·163	0·164	0·166	0·171	0·177

absorption corresponds to about 0·3 % conversion, so that a considerable change is evident in the solutions in 0·4 % ammonia, even after intervals of less than an hour; but those in 0·04 % ammonia are stable over a much longer period.

[1] The transmission of this "Spectrum Orange" filter was found to be very sensitive to heat radiated from the lamp. The resultant error could be avoided by allowing it to cool for about 2 min. between successive readings.

Since in experiments of this type the successive readings are being taken on the same blood, the detection of changes in the blood is not subject to the uncertainty caused by the 5% variation of different blood specimens explained in the previous section.

Table 2 contains similar data for the production of alkaline haematin from methaemoglobin. Readings were taken in this case with the green filter. 1% decrease in absorption now represents roughly 3·5% conversion. Similar results were obtained with other blood samples. Once again, the solutions in 0·04% ammonia are more stable.

Table 2. *Conversion of methaemoglobin into haematin in presence of ammonia*

(Green filter)

Time after dilution with ammonia ...	Ammonia concentration %	8 min.	4 hr.	40 hr.	*p*H measured by H_2 electrode
Sample 1	0·4	0·410	0·350	0·337	
,, 2	0·4	0·412	0·352	0·338	10·84
,, 3	0·1	0·422	0·398	0·372	
,, 4	0·1	0·419	0·395	0·370	
,, 5	0·04	0·419	0·413	0·396	
,, 6	0·04	0·419	0·414	0·398	
,, 7	0·02	0·422	0·418	0·411	
,, 8	0·02	0·424	0·418	0·410	9·86

In a further series of experiments, it was found that the rate of conversion of methaemoglobin is almost independent of the time interval between the preparation of the initial methaemoglobin solution and its dilution with ammonia; but it is slightly influenced by the ratio in which it is diluted.

The results indicate that the use of 0·04% ammonia is safe for most purposes, provided that the solutions are not allowed to stand for too long. Lower concentrations of ammonia are not suitable, since the solutions may become turbid owing to precipitation of blood proteins. Some light is also shed on the recorded inconsistency of spectrophotometric measurements on methaemoglobin at high *p*H values, such as solutions in sodium carbonate [e.g. Denes, 1930; see also Haurowitz, 1924]. The solutions of methaemoglobin in 0·4% ammonia used in the present experiments had *p*H about 10·9, so that readings at higher alkalinity could not be expected to give reproducible results.

The author is grateful to Dr G. A. Harrison for his kind interest in this work. He also acknowledges his indebtedness to St Bartholomew's Hospital Blood Transfusion Station, Luton, for the supply of materials, and to Messrs B. Laporte Ltd., Luton, for kindly providing laboratory facilities.

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174. THE BIOLOGICAL ASSAY OF TESTICULAR DIFFUSING FACTOR

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(*Received 23 September 1940*)

In a series of papers Madinaveitia [1938; 1939, 1, 2; 1940, 1, 2, 3] has recently described investigations into diffusing factors that have been prepared as concentrates, mainly from bull testicle, but also from "Padutin" (Bayer), a commercial preparation of kallikrein, from bacterial cultures, from snake venom and from leech extracts. It has been suggested by Chain & Duthie [1939], and the view has been supported by Favilli [1940] and by McClean & Hale [1940], that diffusing activity may be due to mucinase, which they have found present in similar concentrates.

Confirmation of this view must depend on the possibility of making quantitative estimations of diffusing activity and comparing the results with the mucolytic activities shown by the same materials. The method described by Madinaveitia [1938] for measuring the diffusing activity of his preparations has been stated by him [1939, 2] to be insufficiently precise to permit the rapid progress of his investigations and is thus obviously not suited without modification to the more stringent requirements of comparison with mucolytic activity, which can be determined with some accuracy.

Both for the purpose of such comparisons and also to make possible more rapid advance in concentrating and purifying the diffusing factor itself, it seemed to us desirable, therefore, to attempt an improvement in the method of quantitatively measuring diffusing activity. We accordingly set ourselves the task of so modifying the technique laid down by Madinaveitia as to permit the conduct of biological assays in the strict sense of the term, that is, the comparison of activity with that of a stable standard preparation. Generally speaking, this can be done by means of the "four-point assay"; two doses of the standard and two doses of test material are used, the dose levels, the dose ratio and the number of responses to each dose being chosen so as, if possible, to permit calculation of potency ratios having fiducial limits ("probable limits of error") of between 60–70 % and 150–130 %. Calculation is carried out by the method of Irwin [1937] in a somewhat simplified form.

For the procedure adopted by Madinaveitia reference may be made to his publications. We have investigated a number of the factors likely to contribute to the inaccuracy of the test and have modified the procedure, not only in the light of the information so obtained, but also in certain directions that seemed to us likely to help towards the same end. For example, we have introduced planimetric measurement of the area of bleb, which is traced in Indian ink on large glass microscope slides. We have also paid special attention to preparation of the haemoglobin indicator solution, with a view to eliminating any traces of diffusing factor that might be present in the horse blood, thus reducing to a

minimum both the bleb size produced by the haemoglobin solution alone and its variance.

Inspection of Madinaveitia's curves suggested to us that differences in bleb size due to different doses did not increase materially after 20 min. from the time of injection, and our own observations confirmed this. We have, therefore, for assay purposes made all our measurements of bleb size after that interval.

Exploratory tests with quantities of testicular extract in the ratio 2 to 1 showed that the mean areas of 6 or 8 blebs gave differences that were generally significant. It was considered necessary to attempt further to measure the influence, if any, on bleb size of the position of the injection on the back of the animal and of the order (in time) of injection, as well as to estimate the contribution made by animal variation to the error of the method. Expert statistical advice was accordingly sought,[1] and as a result it was decided to carry out an experiment with six rabbits and six positions for injection on the back of each rabbit and to base the experiment on a Latin square design.

A 6×6 Latin square was therefore selected by the procedure described by Fisher & Yates [1938]. Table 1 illustrates the plan of the experiment. Arabic numerals (1 to 6) refer to the animals, letters (*a* to *f*) to the position on the backs of the animals and Roman numerals (i to vi) to the order of injection. Thus each animal was injected once and once only in each position and each position was the *r*th to be injected (r=i, ii, ..., vi) in only one animal. Also, the sites (*a* to *f*) were so arranged that three of them (*a*, *c* and *e*) lay near the animals' vertebrae, while the other three (*b*, *d* and *f*) occupied more lateral positions. In Table 1 are also shown the actual bleb sizes (in square centimetres) 20 min. after the injection.

Table 1

	Animals					
Positions	1	2	3	4	5	6
a	iii	v	iv	i	vi	ii
	7·9	8·7	7·4	7·4	7·1	8·2
b	iv	ii	vi	v	iii	i
	6·1	8·2	7·7	7·1	8·1	5·9
c	i	iii	v	vi	ii	iv
	7·5	8·1	6·0	6·4	6·2	7·5
d	vi	i	iii	ii	iv	v
	6·9	8·5	6·8	7·7	8·5	8·5
e	ii	iv	i	iii	v	vi
	6·7	9·9	7·3	6·4	6·4	7·3
f	v	vi	ii	iv	i	iii
	7·3	8·3	7·3	5·8	6·4	7·7

The total sum of squares of deviations from the mean of all the observations is 30·3599 and the analysis of variance is as follows:

	Sums of squares		Degrees of freedom		Mean squares	
Animals	12·8333		5		2·5667	
Position	3·8332		5		0·7667	
Order	0·5632	17·5266	5	30	0·1106	0·5842
Error	13·1302		20		0·6565	
Total	30·3599		35		(0·8674)	

[1] The statistician concerned wishes to remain anonymous; we must nevertheless express to him our sincere thanks for his assistance.

The effects of position on the animal and of order of inoculation are not significant, and the last three components of variation may therefore be combined to give an error variance of 0·5842. The estimate of the portion of the total variance due to animal variation is therefore $(2{\cdot}5667-0{\cdot}5842)/6=0{\cdot}3304$.

An unbiased estimate of the total variance is therefore $(0{\cdot}3304+0{\cdot}5842)=0{\cdot}9146$. Of this 36 % is due to animal variation and 64 % to general experimental error. It is also clear that the mean of all the median blebs (positions *a*, *c* and *e*) does not differ significantly from the mean of all the lateral blebs (positions *b*, *d* and *f*); the values are 7·36 and 7·38, with $t=0{\cdot}7$ and $P=0{\cdot}45$.

The result of the experiment shows that it will be advisable, whenever possible, when two different preparations of diffusing factor are to be compared, to inoculate the pairs of doses to be compared into each of several animals, so that animal variation may be eliminated from the error of the result. It is clear that the site of the blebs on the animals' backs—within the range used in the Latin square experiment—and serial position in time have no significant effect and need not be controlled, but that uniform distribution of test doses over all the animals used is essential if the error of a test is to be kept minimal.

The ability to distinguish significantly between the means of six or eight responses to doses in the ratio of 2 to 1 is, however, generally considered a satisfactory achievement in the biological assay of hormones, vitamins and so on, and can be made the basis of four-point assays having not more than the desired fiducial limits.

Investigation of several varieties of rabbit were also made, with a view to using animals giving not only the maximum discrimination between doses in a given ratio but also the smallest variance in response to a given dose. At the same time we examined the range of doses over which it should be possible to conduct "four-point" assays, that is, the range over which the relationship between response and logarithm of dose did not depart significantly from linearity.

In general, it is necessary to use large rabbits, weighing at least 2 kg., having non-pigmented skins (a condition independent of fur colour) fairly, but not excessively thin. We have found no variety better than the Belgian Hare-Flemish Giant cross, although we have used Himalayan, Dutch, Polish and other breeds. Chinchillas are the least satisfactory.

With the animals and the technique outlined, a range of doses giving bleb-sizes from 4 to 8 sq. cm. has been found to satisfy the necessary requirements, the bleb-size given by the indicator solution being always below 4 and generally below 3·5 sq. cm. The mean control bleb-size is about 3·3 sq. cm. and the variance of the indicator bleb-sizes is very low.

The following is a summary of the procedure now used by us:

Suitable rabbits—preferably crosses between Belgian Hare and Flemish Giant, but in any event animals with thin non-pigmented skins—are shaved, with or without previous depilatory treatment (such as application of the paste described by Zacho [1939] for use on guinea-pigs). Almost the whole of the back and sides can be shaved, permitting production of 12 or even more blebs on each animal.[1] Injection is made with a 240 intradermal Agla needle, on the day following shaving.

The indicator solution is prepared from centrifuged fresh horse blood; the corpuscles are washed 4–5 times with fresh isotonic saline. The saline is decanted and distilled water is added to give 100 ml. of solution with the corpuscles from 100 ml. of blood. Laking must be complete, as judged microscopically, and after

[1] One of us (T. R. M.) has recently been able to accommodate 21 blebs on each of four rabbits.

centrifuging there must be no unbroken cells in suspension. This solution will keep in the refrigerator for 2 or 3 weeks. Any indicator solution producing in doses of 0·2 ml. blebs as large as 4 sq. cm. (in 20 min.) should be discarded.

The amount of indicator solution used is always 0·2 ml.; with this is mixed the required dose of diffusing factor, made up to 0·1 ml. with distilled water, giving a total of 0·3 ml. for injection.

Injections should be so arranged that each animal receives the same number for each test dose: with large animals the number can be three, and there will also be room for one or two control blebs. Injections should be so arranged in time that the first blebs to be made do not encroach on the area required for those made later: there is still an increase in bleb-size after 20 min., but there is no difficulty in avoiding overlap with proper arrangement of doses in time and space.

The calculation of variance, standard error of mean bleb-size and potency ratio follows the now well-known procedure of Irwin [1937]. There is, however, a departure from the usual condition, in which means are calculated from a series of observations normally distributed round the mean. As we have found that the differences between bleb-sizes on the same animal are less than the differences between mean bleb-sizes on different animals for the same dose, the distribution of the whole of the observations will depart from normal if marked differences exist between animals, especially with the small number used—three or four. It is therefore the more important to use animals showing as nearly as possible the same mean response. This is best achieved by careful selection for skin-thickness and by having available one or two animals besides those actually to be used in the assay. As soon as the first blebs have been observed, any abnormally sensitive or insensitive animal can be rejected and one or more of the reserves brought into use. We have, however, seldom found this necessary. A suitable modification of statistical procedure, by analysis of variance, would make it possible to deal with any markedly heterogeneous distribution shown by the responses.

Certain experimental records will now be quoted to illustrate the kind of result obtainable.

(1) Two doses of a concentrate were tested; they were in the ratio of 2 to 1. Each dose was used to produce four blebs on each of two rabbits. The results were as follows:

	Bleb area (sq. cm.)	
Animal	Low dose	High dose
1	4·0, 4·5, 5·4, 5·7	6·8, 7·1, 6·8, 7·1
2	4·8, 5·0, 4·2, 5·5	5·8, 6·6, 7·3, 5·9
Mean	4·89	6·68
S.E. of mean	0·220	0·196

For the difference between the means, t has a value of six, corresponding with $P \ll 0{\cdot}001$. Another animal of the chinchilla type was used to produce six blebs with the lower dose; these had a mean area of only 3·7 sq. cm., indicating that the animal was less sensitive than the thinner-skinned rabbits used for the comparison. In this test, doubling the dose produced an increase of 1·8 sq. cm. in mean bleb-size; the value of b, the slope of the response curve, is therefore $\frac{1.8}{\log 2} = 6$.

(2) A powdered concentrate was tested in doses of 1·6 μg. and 3·2 μg. on two rabbits, each receiving three injections of each dose. The mean bleb values were 7·08 and 8·55 sq. cm.; for this difference the value of t was 3·05 ($P = 0{\cdot}015$). The slope of the response curve was 4·85, rather less than in the preceding experiment; ceiling values of diffusing factor were possibly being approached. Polish rabbits

were used in this experiment and they may respond to a somewhat lower dose level than the preferred cross, owing to their smaller size, which is also a disadvantage in itself.

(3) A certain batch of material (*A*) had been used in preliminary tests as a provisional standard preparation: it was desired to replace this with another batch (*B*) of which considerably larger supplies were available. As it was only possible to hazard a guess at the relative potencies of *A* and *B*, it was decided to test the latter at four dose levels compared with two dose levels of the former. The results are shown in Table 2.

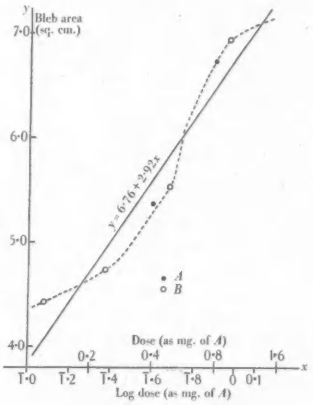

Fig. 1.

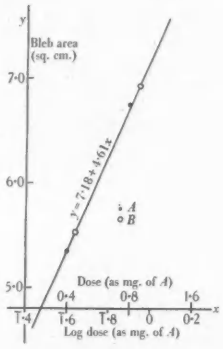

Fig. 2.

Table 2

Sample	Dose μg.	No. of blebs	Bleb area (sq. cm.) Mean	Stand. dev.	Coeff. of var.	s.e. of mean	For differences t	For differences P
A	0·4	12	5·36	0·95	18·0%	$0·27_5$	3·03	0·008
	0·8	12	6·75	1·26	19·0%	0·36		
B	0·1	12	4·42	0·64	14·5%	$0·18_5$	1·13	0·25
	0·2	12	4·74	0·75	16·0%	$0·21_5$	2·08	0·05
	0·4	12	5·52	1·06	19·0%	$0·30_5$	3·40	0·003
	0·8	12	$6·92_5$	0·95	14·0%	$0·27_5$		

Table 3

Assay calculated from	Total no. of blebs	Slope of combined dosage-response curve	Potency ratio *B* : *A*	Fiducial limits of dose ratio $P=0·95$	Fiducial limits of dose ratio $P=0·99$
2 doses *A*	72	2·92	$1·20_5$	0·82–1·78	0·72–2·01
4 doses *B*	—	—	—	(67·5–148%)	(59·5–167·5%)
2 doses *A*	48	4·61	$1·09_5$	0·81–1·48	0·74–1·62
2 doses *B*	—	—	—	(74–135·5%)	(67–149%)

If the responses are all taken into account and the potency ratio is calculated by means of a dosage-response curve derived by the method of least squares, a result is obtained that is shown graphically in Fig. 1. The value of *b*, 2·92, is

much less than found in Exps. 1 and 2. Moreover, it is clear that the departure from linearity of the six plotted points indicates that the sigmoid nature of the dosage-response curve has persisted even when response is plotted against log dose. This phenomenon is discussed by Emmens [1940] in a forthcoming publication; it shows that the range of responses found is too wide to justify them all being used to construct the regression line for response on log dose. Accordingly the result of the assay has been recalculated, omitting the responses to the two lower doses of *B*. Graphically, this is shown in Fig. 2. Linearity is here completely restored, because the four responses fall on that part of the dosage-response curve where response is a linear function of log dose.

If the two assays—"six-point" and "four-point" respectively—be calculated according to Irwin's method, results are obtained as shown in Table 3. It is clear, as the plotted curves also indicate, that reducing the number of observations by one-third—that is, eliminating those that invalidate calculations by the method of least squares—has not only brought about some change in the found potency ratio, but has also considerably reduced the error of the test. Obviously, had 72 blebs been available for use in the "four-point" assay, the fiducial limits would have been still further reduced. It will be noted that the "four-point" assay gives to b a value of 4·6, rather less than that obtained in Exp. 1, but not significantly different from that obtained in Exp. 2.

(4) In this experiment we assayed a sample of Dr Madinaveitia's standard preparation (*M*), of which he very kindly supplied us with a sample. Having no foreknowledge of its potency in relation to our own standard, we tested both at three dose levels, using five rabbits, with the results shown in Table 4.

Table 4

Sample	Dose μg.	No. of blebs	Bleb area (sq. cm.) Mean	Stand. dev.	Coeff. of var.	S.E. of mean	For differences t	For differences P
M	0·2	10	4·25	0·80	18·7%	0·25		
	0·4	10	4·85	0·96	19·8%	0·30	1·5	0·15
	0·8	10	5·93	0·76	12·9%	0·24	2·8	0·01
B	0·2	10	5·04	1·12	22·3%	0·36		
	0·4	10	5·47	0·89	16·2%	0·28	0·95	0·35
	0·8	10	6·45	1·15	17·9%	0·36	2·2	0·04

Table 5

Sample	Total no. of blebs	$\bar{y}_2 - \bar{y}_1$	Mean variance	b	Units (G.L.)* per mg.	Fiducial limits $P=0·95$	Fiducial limits $P=0·99$
A	4×12	0·17	1·134	4·61	1820	74–135·5%	67–149%
M	5×8	0·57	0·905	3·42	1370	63·5–157%	55–181%

* For assay purposes a "unit (G.L.)" is defined as the diffusing activity of 0·5 μg. of product *B*.

It is clear that the lowest dose of each standard is below the threshold, that is, gives a response on the tail of the dosage-response curve, where response is no longer proportional to log dose. These two responses were therefore ignored, and the potency ratio of *M* and *B* was calculated from the remaining four responses. For purposes of comparison, the results are shown in Table 5 along with the results of the evaluation of our first laboratory standard (*A*) in terms of the second, as described under Exp. 3.

It will be seen that the assay of *M* was rather less satisfactory than that of *A* (against *B* as standard in both instances). This is largely due to a low value of b,

for which the relatively poor discrimination shown between doses was responsible: doubling the dose—from 0·4 to 0·8 μg.—produced an increase in mean bleb size of only 1·03 sq. cm., whereas in Exp. 3 the same increase in dose had produced an increase of 1·4 sq. cm. in mean bleb-size.

(5) It was desired to examine the effect, if any, of including 0·5 % of phenol on the diffusing factor activity of an aqueous solution of a solid concentrate. A four-point assay, using the usual dose ratio of 2 to 1, was carried out on four animals, each of which received two injections of each dose. The results are shown in Table 6.

Table 6

		Bleb area	
Solution	Dose	Mean	$(S.D.)^2$
Without phenol	Lower	5·81	0·781
	Higher	$7·42_5$	0·197
With phenol	Lower	5·45	0·262
	Higher	7·24	0·344

A very low mean variance was found in this assay (the mean coefficient of variation was only 9·7) and this, combined with a high value of b (5·64) led to very low fiducial limits for the mean potency ratio calculated. The figures were

	Probable limits of error	
Ratio	$P=0·95$	$P=0·99$
0·894	79–127 %	83·5–120 %

In spite, however, of the low limits of error, it was found that the ratio of 0·894 did not depart significantly from 1—as can be seen from the values for $P=0·95$; the chance of the 10 % difference being due to random sampling errors is 1 in 6.

These experiments seem to us to indicate that the level of accuracy obtainable with the technique described is not on the average less than that of other biological assays carried out on a comparable scale. There are, of course, variations from test to test, both in the values of b and in the mean variance of response, but such fluctuations in the gamble of animal assay are inevitable and can be urged no more cogently against one procedure than against another; the crucial test is the general level of error found over a series of assays. In this connexion it is pertinent to point out that bleb-size shows in various dosage groups a coefficient of variation ranging from 5·9 to 22·3 % with a mean (unweighted) value for 22 groups (216 blebs) of 14·3. A scattergraph shows little evidence of correlation between mean bleb size and the standard deviation of single observations in a group; the correlation coefficient for the two sets of values was indeed found to be only 0·35 (for 22 pairs, $P=0·1$), suggesting that any other expression of diffusing effect—e.g. square root of bleb-size or logarithm of bleb-size—is unlikely of itself to lead to a reduction in the error of tests carried out by our present procedure.

Summary

A method is described for comparing the diffusing factor activities of pairs of preparations, with an estimate of the error of comparison, by measurement of the mean bleb-size produced in suitable rabbits, followed by the construction for each assay of a response-log dose curve by the method of least squares and calculation of the fiducial limits of the derived potency ratio of the preparations. The error is no greater than that found in other biological assays carried out and evaluated on comparable lines.

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175. STUDIES ON THE "FERMENTATION" OF CEYLON TEA

1. THE NATURE OF THE ENZYME SYSTEM

2. OXIDIZING ENZYMES

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(*Received 4 September 1940*)

1. THE NATURE OF THE ENZYME SYSTEM

"Fermentation", as understood in the tea industry, is the process during which "black tea" develops its characteristic aroma and colour of liquor. The development of black tea characteristics may be arrested by steaming freshly gathered tea leaf and this device is employed in making green tea. It is at once fairly obvious therefore that fermentation is an enzymic process and this has been confirmed by several workers [Gadd, 1932; Benton, 1938]. Benton [1938] has made an intensive study of secondary effects in fermentation due to micro-organisms. The tea bushes in cultivation at present are not anywhere much more than a selection from a thoroughly heterogeneous collection of accidental hybrids, and the conditions of cultivation are so diverse, ranging from tropical to almost temperate, that we wish to make it clear that we are not attempting to explain anything more than our own observations in Ceylon, so far confined to leaf grown at high elevations (4500 ft.).

However, we cannot pass on to an account of our investigations in Ceylon without some reference to work in other countries which has been principally concerned with three classes of enzymes, namely, oxidases, peroxidases and catalases, and their function in the main reaction which appears to be the oxidation of polyphenols. Thus Nanninga [1901], Aso [1901], Newton [1902] were the earliest to describe the enzyme as oxidase (thease) and Mann [1901], Bernard & Welter [1911], Kursanov [1935], and Oparin [1935] showed the presence of both oxidase and peroxidase. More recently Roberts & Sarma [1938] have realized the interest of investigations on tea fermentation "to the biochemist, not only as an illustration of the growing importance of enzyme chemistry in industry, but also as a detailed study of the chemical changes taking place in damaged plant tissue". These workers studied a peroxidase system in Assam leaf but were unable to detect oxidase, the latter observation, however, having been qualified in a joint letter [Lamb & Roberts, 1939].

Roberts & Sarma [1938] state that "various qualitative studies have been made at the several Tea Research Institutes, but no thorough investigation has been made apart from that of Oparin [1935]". Quantitative studies have actually been in progress in Ceylon since Evans [1927] first investigated the absorption of O_2 by fermenting tea leaf. Evans found that leaf dipped in 1% $HgCl_2$ before crushing fermented quite normally and in this way ruled out micro-organisms; he then proceeded to measure O_2 uptake and found that rapid

absorption of O_2 occurred during the first 3 or 4 hr. of fermentation after which the rate decreased. From the figures obtained Evans was able to plot a smooth "die-away" curve characteristic of enzyme reactions. The rate of O_2 absorption was also unaffected by the presence of hydrogen cyanide, an observation which is of considerable interest in connexion with the role of peroxidase, marked quantities of which enzyme are present in Ceylon leaf. Catalase was also studied by the same worker who found that catalase activity declined rapidly during the fermentation process but he was unable to interpret the significance of his findings. The present investigation in Ceylon started in 1936, brief progress reports having been published [Lamb, 1936; 1937].

Fermentation of tea in Ceylon

In Ceylon, tea is pruned down every 2 to 4 years according to elevation and climatic factors, thus maintaining the bushes in a state of active vegetative growth. The buds with two partially matured leaves ("flush") are harvested at intervals of 7–12 days, and taken to the factory where:

(1) The flush is slowly withered to 50–55 % moisture content by means of conditioned air, or natural breezes. The operation normally takes about 18 hr. and the leaf becomes flaccid.

(2) The withered leaf is then rolled in lots of 200–400 lb. in machines which apply a twisting motion. The effect on the leaf may be likened to the wringing of a damp cloth. During this process some of the juices are worked out of the leaf and form a varnish-like coating on the surface. Fermentation is started by the rolling process which is normally continued in successive stages of 30 min. The leaf is sifted after each stage until 80–90 % of the leaf is twisted (and broken) into a size which will allow passage through standard sieves.

(3) The leaf sifted from the rolling process is spread in layers 2 in. thick, usually on cement slabs, to continue fermentation, which is timed from the commencement of rolling and ranges from $1\frac{1}{2}$ to 4 or more hours.

(4) Subsequent stages involve drying with hot air at 85–95° to a moisture content of 3 %, cleaning, grading etc.

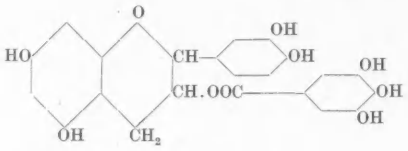
The substrate

Again, it is necessary to emphasize the fact that tea bushes in cultivation at present cannot by any test be considered as a pure line crop. The observations which follow cover only the material gathered from our own estate. Although selection work is in active progress it has not so far been found possible to work with clonal material. It has, however, been practicable to manufacture the leaf from single bushes and these investigations have given ample proof of the heterogeneity of the material in cultivation on our own estate [Lamb, 1939]. The polyphenols which have received the most attention may be roughly divided into three classes: (*a*) tannins, (*b*) catechins, (*c*) simple polyphenols. Tsujimura [1929] considers the principal tannin to be the galloyl ester (I) of a tea catechin (II). Deijs & Dijkman [1936], working with Java tea, produced evidence in support of this structure and succeeded in splitting off gallic acid by means of *Aspergillus niger*. Oshima [1936] succeeded in synthesizing a tannin (III) which he claims to be identical with the natural product of Formosan leaf. In Ceylon we have not succeeded in splitting gallic acid from the amorphous tannin preparations from our leaf. The culture of *A. niger* used for the purpose split gallic acid from commercial white tannin and grew vigorously on our tea tannin

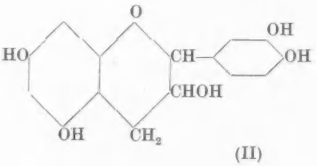
preparation, but without producing any gallic acid. Ceylon leaf, however, contains marked quantities of gallic acid in an apparently free state. It seems quite probable that at least two different tannins may occur in tea, but under our conditions the galloyl ester type does not appear to be present in any marked quantity.

The two principal catechins in Ceylon leaf appear to be *l*-epicatechin (II) also called tea catechin [Tsujimura, 1929] and gallo-catechin (IV) of Oshima. The quantities, however, are small compared with the tannins. Deijs [1939, 1, 2] has recently isolated these catechins from Java leaf.

Of the simple polyphenols, gallic acid, as mentioned above, occurs in marked quantities. Catechol is also probably present in traces:



(I)



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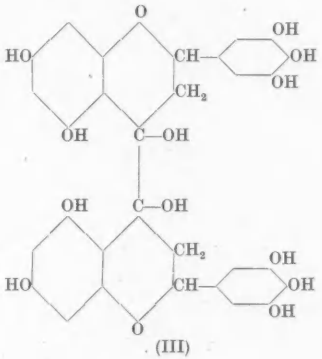

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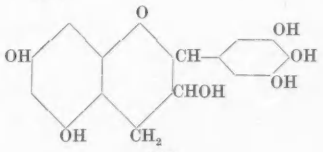

(IV)

Reference must also be made to some of the other constituents of Ceylon tea leaf. In view of the work of Roberts & Sarma [1938] it is necessary to mention that ascorbic acid[1] is present in amounts varying between 0·2 and 0·4 mg. per g. of fresh weight. Sugar in the various parts of the flush ranges from 1·41 to 0·73 % of dry weight, and starch, which does not clear at night from the leaves of some varieties, ranges from 2·96 to 0·82 % of dry weight. Some chlorophyll degradation takes place during fermentation and is of importance in connexion with the formation of the insoluble pigments of made tea as will be shown later. Caffeine, which will precipitate some of the polyphenolic constituents of tea leaf [Shaw, 1935], occurs to the extent of 3·5 % of the dry weight of the average sample of Ceylon tea leaf.

[1] Estimated by means of 2:6-dichlorophenolindophenol and Drumstick ascorbic acid oxidase [Sreerangachar, results prepared for publication].

The enzyme system

On crushing either fresh or withered Ceylon tea leaf the development of a coppery red colour commences in the crushed tissue exposed to the air, and within a period of roughly 4 hr. almost all the initial green colour of the crushed leaf has changed to a coppery red tint. Extraction of different portions of such crushed leaf with water at intervals shows intensification of colour in the aqueous extract from an initial greenish-lemon to a deep reddish brown, whilst the extracted tissue retains an insoluble pigment which is initially pale yellowish green and finally a bright coppery red. The final extract is well illustrated by the liquor from the ordinary domestic brew of tea and the insoluble pigment by the colour of the spent leaves or "grouts".

Addition of H_2O_2 to the crushed leaf causes a very marked change of colour in a short period, consistent with the activation of a peroxidase. The crushed tissue will liberate O_2 from H_2O_2. If fresh or withered leaf be heated above 120° F it remains green after crushing. Fermentation is also inhibited by preventing access to O_2. CO_2 continues to be evolved by crushed withered leaf, but at a greatly reduced rate compared with uncrushed fresh or withered leaf [Evans, 1928].

EXPERIMENTAL

In order to save space, much experimental detail must be condensed but we shall endeavour to give a logical summary of the successive stages of our work and to enlarge only upon the more important points.

(1) *Preparation of enzyme system.* A weighed quantity of fresh or withered leaf is ground with iron-free sand under acetone and rapidly filtered under suction, repeating the operation until the coloured constituents are leached out. The greenish white residue is then dried *in vacuo*. McIlvaine's or Sørensen's phosphate buffer may be employed to extract soluble enzymes from the resultant dry powder, for which purpose one extraction of 30 min. is sufficient. The extract gives a faint ferric chloride test.

(2) *Preparation of substrate.* A weighed quantity of undamaged fresh or withered leaf is immersed in boiling water for 5 min. in order to inactivate enzymes. The material is then drained and the juices expressed and stored in a non-actinic bottle under toluene. This stock solution keeps for at least 14 days. Stock solution may be diluted with McIlvaine's or Sørensen's phosphate buffer as required.

(3) *Quantitative method for measurement of oxidizing activity.* 10 ml. of reaction mixture consisting of substrate solution diluted with buffer, and in some cases enzyme extract, are mixed with 20 ml. $N/10$ I_2 and 12·5 ml. N NaOH. The mixture is then kept in the dark for 15 min. and back-titrated with $N/20$ $Na_2S_2O_3$ after the addition of 25 ml. of 1·5 N H_2SO_4, using a starch indicator. The method is essentially that of Shaw [1935] and is reliable for the principal tannin of tea, and may be used to follow oxidative changes taking place during fermentation. Its advantages are speed and definition of end point, together with a degree of simplicity and directness which safeguards against the confusion which may arise from precipitation methods.

The limitations of the method must, however, be borne in mind. It is not in any way specific, even for polyphenols, and does not afford any indication of changes such as condensation. The method can only be regarded as a measure of changes in oxidizable material. However, in the enzymic reactions studied, the changes in titre have been directly proportional to O_2 absorption value as will be shown in § 12.

(4) In vitro *reaction with soluble enzyme.* Both quantitative and qualitative reactions are carried out in a series of boiling tubes through which washed air may be bubbled. The bubbling also serves to keep all reaction mixtures well stirred. Controls for autoxidation are similarly aerated, the enzyme preparation, if in solution, being in each case replaced by an equal volume of buffer solution. For most quantitative experiments the reaction is allowed to proceed for 4 hr., and if it is desired to observe peroxidase activity, 0·25 ml. portions of $N/10$ H_2O_2 solution are added at suitable intervals. A total volume of 25 ml. is normally convenient to work with, a typical reaction mixture for observation of soluble enzyme activity being 10 ml. of enzyme solution, 10 ml. of substrate solution diluted 5 times with buffer and the whole made up to 25 ml. The relative amounts of enzyme and substrate may be arranged so that they are both equivalent to a given amount of fresh leaf.

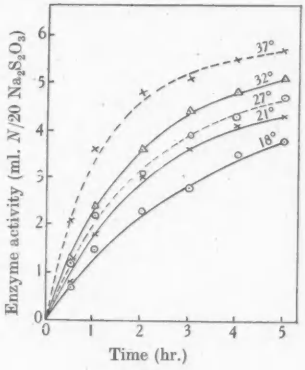

Fig. 1.

Soluble enzyme preparations are sensitive to high concentrations of tannin and are practically inhibited by the undiluted substrate solution. Soluble enzyme activities are therefore observed in diluted substrate. The insoluble enzyme preparations described later are fully active in undiluted substrate. The addition of H_2O_2 causes an increase in enzyme activity measured by titration. In a typical case the titration difference increased from 3·7 to 5·0 ml.

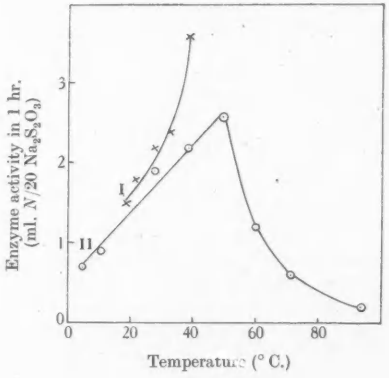

Fig. 2.

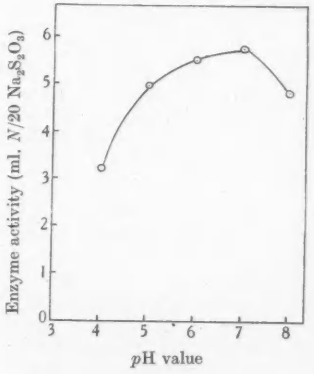

Fig. 3.

(5). *Kinetics,* p*H and temperature in relation to soluble enzyme.* Fig. 1 shows oxidizing activity of the soluble enzyme plotted against time in hr., over a temperature range of 18–37°. Fig. 2 shows that the optimum temperature for oxidation in the enzyme-substrate system prepared from Ceylon tea leaf is in the region of 49°, and Fig. 3 indicates that the optimum *p*H for the same system

is between 6·0 and 7·0. In all three cases corrections have been made for autoxidation of substrate.

(6) *Purification of soluble enzyme.* The soluble enzyme system is precipitated from buffer solution by 80 % alcohol. Half saturation with ammonium sulphate also brings about precipitation of active material, but in neither case is the full activity of the enzyme system preserved. The juice of fresh leaf may also be treated with acetone or alcohol to precipitate a similar white gelatinous mass which shows marked activity. This precipitate is partly soluble in water.

Ordinary dialysis and ultrafiltration revealed that part of the enzyme system is apparently of low molecular weight since it passes through collodion and parchment membranes, the ultrafiltrate containing active enzyme. Attempts at absorption on alumina C and kaolin have failed, the solution of the enzyme system retaining its full activity. On heating a concentrated solution of the active enzyme system in water there is no indication of any heat coagulation. These last two observations support the suggestion that the activity of the system is due to substances of low molecular weight. In our hands attempts to crystallize active materials from the active solution have so far failed.

A search for accelerators at the time of the dialysis experiments gave some indication that Cu and Mn together have some accelerating effect on the enzyme system. This observation requires further investigation in view of the fact that certain Nyasaland [1937] teas which failed to ferment properly were induced to behave normally by spraying with copper solutions. Keilin & Mann [1938] have described a polyphenol oxidase which consists of a copper-protein complex.

(7) *Mechanism of the oxidation of polyphenols by the soluble enzyme system.* Purified enzyme was prepared by the following method. 100 g. fresh leaf were minced and mixed with 100 ml. water at *p*H 6·5. This mass was then pressed out and the juice precipitated by adding alcohol to a concentration of 80 %. The precipitate was filtered, washed with 80 % alcohol and dispersed in water. This suspension was then filtered and the filtrate made up to 50 ml. The solution was almost water-white when fresh but discolored on keeping for 4–5 days. It gave a very faint reaction with ferric chloride.

Pugh & Raper's work [1927] with tyrosinase suggested to us the investigation of the action of the enzyme system upon catechol. Following their technique we were able to isolate dianilino-*o*-benzoquinone (M.P. 190°; N found 9·78 %; calc. for $C_{18}H_{14}O_2N_2$ 9·66 %).

Subsequently anilinoquinones were prepared from Gambir catechin isolated from B.P. catechu and Tsujimura's tea catechin isolated from Ceylon leaf. The difficulties of crystallization were, however, such that by the time clean crystals were obtained the quantity was insufficient for analysis. White amorphous tea tannin and gallocatechin are also oxidized by the enzyme and in the presence of aniline gave a cumulative precipitate during the reaction.

The progressive formation of precipitates when the reactions are carried out in the presence of aniline is considered to be an indication that quinones are formed, which indication is supported by the observation (*vide* following paper) that the oxidation product of amorphous tea tannin oxidizes ascorbic acid. Quinones formed during these reactions are either unstable or themselves powerful oxidizing agents since complex reactions follow their production usually with intense colour formation.

Within the limits imposed by the difficulties explained above we were able to investigate the specificity of the soluble enzyme system, the results being set out in Table I.

Table 1

Substance	Nature of substance	Reaction (2–3 hr.)
Monohydric phenols:		
Phenol	—	None
Cresols	*o*- and *p*-Hydroxytoluene	None
Tyrosine	*l*-*p*-Hydroxyphenylalanine	None
Dihydric phenols:		
Catechol	*o*-Dihydroxybenzene	Quinone formation
Guiacol	Ditto with one OH group methylated	None
Diacetyl catechol	Fully acetylated catechol	None
Resorcinol	*m*-Dihydroxybenzene	None
Quinol	*p*-Dihydroxybenzene	None
Orcinol	*m*-Dihydroxytoluene	None
Adrenaline	*o*-Dihydroxybenzene with side chain	Very rapid oxidation
Trihydric phenols:		
Pyrogallol	1:2:3-Trihydroxybenzene	Oxidation
Phloroglucinol	1:3:5-Trihydroxybenzene	None
Hydroxymonocarboxylic acids:		
Protocatechuic acid	3:4-Dihydroxybenzoic acid	Oxidation
Gallic acid	3:4:5-Trihydroxybenzoic acid	Oxidation
Nadi reagent	α-Naphthol and *p*-phenylenediamine	Very slight oxidation

The results set out in Table 1 show that the enzyme system is highly specific in that it oxidizes polyphenols having hydroxyl groups in the *o*-position only. Very careful tests failed to give any indication of oxidation of monophenols and, unlike peroxidase, the system showed no sign of activity with *p*-hydroxyphenol (quinol). The slight but definite oxidation of Nadi reagent may be attributed to the action of traces of the quinones of polyphenols associated with the enzyme, as is more fully explained later (*vide* following paper). The soluble enzyme system oxidizes all the catechin and tannin preparations so far obtained from Ceylon tea leaf and there is little reasonable doubt that the first stage of the oxidation is the production of an *o*-quinone. This statement is consistent with the constitutional formulae depicted in the section above dealing with the substrate.

Since ascorbic acid is eliminated by the method of preparation of the enzyme system used in studying the specificity there cannot be any question of the formation of H_2O_2 by an ascorbic acid–ascorbic acid oxidase system. Furthermore, it appears that peroxidase activation does not take place since peroxidase is not so specific in action. Further investigation of these reactions, along the lines recently followed by Jackson [1939] in his most interesting studies of oxidation by polyphenol oxidase, is desirable.

(8) *The insoluble enzyme system.* Having gathered information about the characteristics of the soluble part of the enzyme system we next directed attention to the residual activity of the preparation after removal of soluble enzyme. Repeated and thorough extractions with several aqueous solvents such as buffer solutions of different *p*H and glycerol did not render the residue inactive. Accordingly, quantitative determinations were made on different samples before and after extraction.

Mention has been made of the heterogeneity of the tea crop cultivated in Ceylon. Careful repetition of all major experiments is thus necessary and the variations encountered are well illustrated in Table 2, where figures obtained from 4 different samples are given. For each determination 0·5 g. of enzyme preparation was taken and the same quantity of stock substrate used in each reaction.

Table 2

System	Activity (ml. $N/20$ $Na_2S_2O_3$)			
	Sample I	Sample II*	Sample III	Sample IV
Unextracted powder	25·25	24·4	52·0	9·0
Extract	18·0	23·2	8·8	12·8
Residue	32·25	41·6	65·6	17·2

* Triplicates agreed to within 0·6 ml.

The stock substrate was not necessarily the same stock; it has to be freshly prepared from time to time. The determinations for any one sample were, however, all carried out with the same stock solution.

Despite the variations in the figures, the same general conclusion may be drawn from the results with each different sample that the residue retains a very high degree of activity which actually appears to be enhanced by extraction of the soluble enzyme. In these experiments the residue was dehydrated with acetone before determination of its activity. A further series of experiments was next carried out to determine the effect of successive extractions with buffer solution. Although the enzyme preparation is relatively tannin-free it actually contains titratable amounts of polyphenols and there was at least a possibility that such substances might act as inhibitors of insoluble enzyme activity. Table 3 shows the result of these extraction experiments. Activities were determined on 10 ml. of a stock substrate solution sufficient in quantity to cover the whole experiment. The wet residue plus filter paper was in each case introduced into 10 ml. buffer solution at *p*H 5·4 before addition to 10 ml. of substrate solution. For each extraction 20 ml. of buffer solution were used and after filtration 10 ml. were titrated in order to determine the amount of oxidizable material extracted.

Table 3

Figures represent ml. $N/20$ $Na_2S_2O_3$.

No. of extractions	Oxidizable material in 10 ml. extract		Activity of 0·25 g. of enzyme preparation
Nil	—		14·2
1		5·6	13·6
2	1st	5·8	15·0
	2nd	1·8	
3	1st	5·1	14·0
	2nd	2·1	
	3rd	0·5	
4	1st	5·6	15·6
	2nd	2·0	
	3rd	0·3	
	4th	0·1	

The figures in Table 3 confirm the fact that a high degree of activity is retained by the residue after successive extractions but do not afford any definite evidence of activation by removal of inhibiting substances.

A separate experiment was carried out at *p*H 5·4 (with different material) in order to study the effect of successive extractions upon the amounts of soluble enzyme removed from the enzyme preparation. The figures are given in Table 4.

If, after the reaction, the residue containing insoluble enzyme is washed free of colour and added to further substrate, it is observed that activity is retained by the residue. This process has been repeated three times and after oxidizing

Table 4

Extract	Activity of extract (expressed in ml. $N/20$ $Na_2S_2O_3$)
1st	12·0
2nd	6·5
3rd	3·0
4th	1·0
Activity of residue	25·5

three successive quantities of substrate, the residue was found still to possess the power of oxidizing further substrate, thus indicating the insolubility of the enzyme system in a buffered solution of its natural substrate. The extractability of the soluble enzyme system from the dry powdered enzyme preparation varies markedly with the *p*H of the buffer solution employed for extraction.

Table 5 displays figures which are a measure of activities resulting from different extractions. Since the optimum *p*H for the oxidizing activity of the soluble enzyme system in tea extract substrate is between 6·0 and 7·0, both *p*H of extraction and *p*H of activity have to be taken into account. The solubility rises markedly between *p*H 5·0 and 8·0, and an extract made at *p*H 8·0 shows very high activity in tea extract substrate at about the optimum *p*H for the system (6·5).

Table 5

	Activity in ml. $N/20$ $Na_2S_2O_3$ per equiv. of 1 g. enzyme powder		
Column I *p*H of extraction and/or reaction	Column II Extraction and reaction *p*H as in column I	Column III Extraction *p*H 7·0 Reaction *p*H as in column I	Column IV Extraction *p*H as in column I Reaction *p*H 6·0–6·5
5·0	31·2	59·4	20·4
6·0	32·4	65·4	30·0
7·0	46·8	68·4	56·4
8·0	61·4	57·6	91·2

The removal of soluble enzyme does not appear to affect the amount of insoluble enzyme activity in the residue of the enzyme preparation. It might be expected that extraction by buffer at *p*H 8·0 would remove most of the activity of the preparation. However, Table 6 shows that the activity of the residue is unaffected by the amount of soluble enzyme removed, since the residue is just as active after successive extractions at *p*H 8·0 as it is after a single extraction at *p*H 5·0.

Table 6

Aliquot no.	Extraction	Activity of insoluble enzyme in tea extract substrate at optimum *p*H 5·4 (*vide* § 10) (ml. $N/20$ $Na_2S_2O_3$)
1	One at *p*H 5·0	6·7
2	One at *p*H 8·0	6·5
3	Four successive extractions at *p*H 8·0	6·2

The relation between the soluble and insoluble parts of the enzyme preparation we have described has caused us much thought and investigation. We intend to refer to only one of many possible explanations, and this arises from an observation that the soluble enzyme activity apparently increases during the process of

withering Ceylon tea leaf. It seemed possible that some process such as cellular lysis might cause the formation of soluble enzyme from insoluble enzyme. Table 7 gives the details of these observations.

Table 7

Preparation	Activity (ml. $N/20$ $Na_2S_2O_3$)
Soluble enzyme from 1 g. of enzyme preparation made from a sample taken from a quantity of well-bulked fresh leaf (moisture content 76%)	25·6
Similar preparation from another sample taken after withering to 63·2% moisture content in 18 hr.	43·2
Another sample withered to 46·4% moisture content in 18 hr.	57·6

The increase of soluble enzyme activity during withering has been confirmed by several experiments and was reported by one of us [Lamb, 1936]. Similar observations in other countries have been reported by Mann [1901] and Jones [1937–8]. Roberts & Sarma [1938], and Deijs [1939, 1, 2] have observed an increase in peroxidase activity. Similar determinations of the activities of insoluble enzyme before and after withering reveal little change; if anything, a slight tendency to increase. The figures in Table 3 show that extraction of soluble enzyme tends to activate the insoluble part of the system. This makes it very difficult to say whether the increase of soluble enzyme during withering is at the expense of the insoluble system.

While the whole question of solubility of the oxidizing enzyme system of Ceylon leaf requires further investigation, we regard it as a problem subsidiary to our main investigations and have pressed on, intending to return to it at a later date. We have, however, investigated the possibility of proteases causing changes in the solubility of these oxidizing enzymes. Papain did not cause the formation of any soluble enzyme from the insoluble enzyme system but, on the other hand, inhibited the oxidizing activity of the preparation.

Roberts & Sarma [1938] have stated that fermentation of finely minced tea leaf suspended in water is inhibited by the addition to the suspension of a liver catalase preparation. They attribute this effect to decomposition of H_2O_2 and consequent effects upon peroxidase activity. No details of the catalase preparation are given, but if it contains any protease there is an alternative explanation of their observations.

(9) *Endo-enzyme.* One of us [Sreerangachar, 1939] has referred to the insoluble enzyme system as endo-enzyme. In the light of subsequent results we have formed the opinion that the term endo-enzyme can only serve to confuse and have accordingly adopted the term "insoluble enzyme" to express the same meaning.

(10) *Optimum pH and temperature of insoluble enzyme activity.* The optimum *p*H and the optimum temperature for the oxidizing activity of the insoluble enzyme in tea extract substrate are widely different from the optima of the soluble enzyme system. Figs. 4 and 5 show these optima to be at *p*H 5·4 and 27°. The latter figure is remarkably low for optimum enzyme activity. The widely different optimum temperatures may explain some of the curious differences in activities recorded in § 8.

(11) *Mechanism of the oxidation of polyphenols by the insoluble enzyme system.* The insoluble enzyme system exhibits the same specificity for *o*-dihydric phenols as the soluble enzyme described in § 7. With *p*-cresol, however, there is a very slight activity more suggestive of impurities than of a definite reaction such as

may be observed with polyphenols containing *o*-hydroxy groupings. Slight oxidation of Nadi reagent was also observed. The anilinoquinone of catechol was again isolated and identified.

Between the soluble and insoluble enzyme systems there is, however, one most important difference. Whereas soluble enzyme tends to be inactivated by

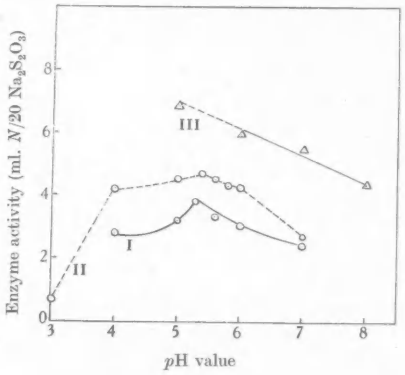

Fig. 4.

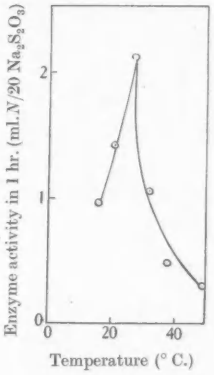

Fig. 5.

high concentrations of substrate the insoluble enzyme remains almost fully active. White amorphous tea tannin preparations moistened with a few drops of buffer are oxidized by the insoluble enzyme when stirred into a paste.

Some evidence of the adsorption of polyphenol by the enzyme preparation was obtained by shaking substrate solution with the powdered enzyme preparation under anaerobic conditions at a low temperature and leaving for 1 hr. On filtering at the end of this period there was a fall in the titre of the substrate solution. Adsorbed tannin may be washed out by buffer solution with some difficulty. Heat-inactivated enzyme preparation has the same property of adsorbing substrate from solution. Glutelin, salt-soluble proteins, starch and pectins were each separately extracted from enzyme preparations without any considerable effect upon the adsorptive power of the residue. In measurement of activity, errors due to the absorptive power of the enzyme powder have been avoided by obtaining initial titration values after the addition of enzyme to the reaction mixture.

(12) *O_2 absorption.* A pair of simple respirometers, illustrated in Fig. 6, were adapted to measure O_2 absorption during reactions between the enzyme systems and various substrates. This particular type of respirometer is very limited in its application but careful work afforded evidence useful in confirming previous findings. It is hoped later considerably to extend investigations in this field with a more sensitive and adaptable apparatus of the Barcroft-Warburg type.

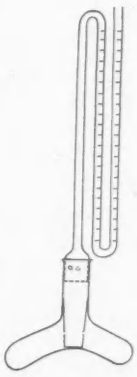

Fig. 6. Respirometer.

First, we were able to confirm the fact that CO_2 is not evolved as a result of reaction between the soluble or insoluble enzyme system

and either tea extract substrate, amorphous tea tannin or simple polyphenols. Secondly, the apparatus afforded a means of establishing the enzymic nature of the O_2 uptake.

Fig. 7 consists of four curves plotted from data obtained on various occasions when *different* enzyme and substrate preparations have been studied at 27° (thermostat).

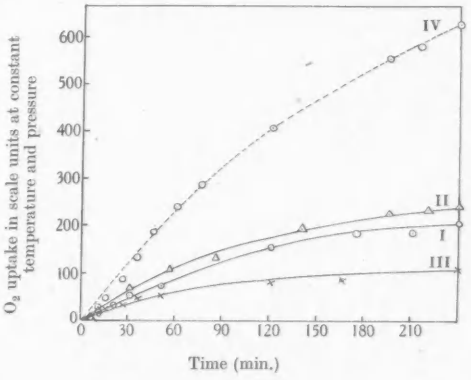

Fig. 7.

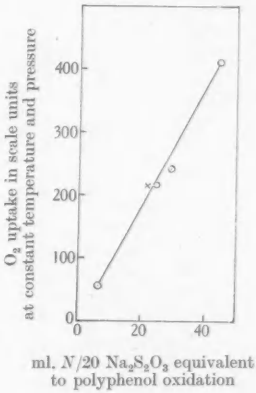

Fig. 8.

Fig. 7. I. 2 ml. concentrated tea extract substrate + 0·3 g. insoluble enzyme powder. II. 3 ml. tea extract substrate + 0·3 g. unextracted enzyme powder. III. 2 ml. amorphous tea tannin solution (1·41%) + 0·3 g. insoluble enzyme. IV. 3 ml. 1% catechol solution + 0·3 g. unextracted enzyme powder.

The slow diffusion of O_2 into the wet tissue makes it impossible to extract any information about the relative activities of these various systems although it has been shown that slowness of O_2 uptake is accompanied by slowness of titrimetric changes and that there is a fairly constant relation between the two methods of observing activity. This relation is shown in the third column of Table 8, and in Fig. 8.

Table 8

Substrate	Enzyme	Ratio O_2 absorbed to change in titre
Tea extract	Mixed enzymes*	8·6
,,	,,	8·1
,,	,,	9·1
,,	Insoluble enzyme	8·6
Amorphous theotannin solution	Mixed enzyme	9·6

* I.e. unextracted enzyme powder.

These experiments have not taken any account of the CO_2 evolved by fermenting leaf but show clearly that O_2 absorption and tannin oxidation may proceed independently of CO_2 production in the primary oxidation processes involving polyphenols.

Evans [1928] studied the evolution of CO_2 by fermenting Ceylon tea leaf and gives the following figures (Table 9):

Table 9

System	ml. CO_2 produced by 40 g. leaf in 1 hr.	ml. O_2 absorbed by 40 g. leaf in 1 hr.	Ratio CO_2/O_2
Fresh leaf	13·9	17·4	0·8
Fresh leaf crushed	14·1	17·6	0·8
Leaf withered to 55%	9·8	11·0	0·9
Leaf withered to 55% and crushed	3·4	26·0	0·13

The same worker showed a slight decrease in the value of CO_2 produced by rolled leaf in each consecutive hr. over a period of 7 hr., the amounts being 4·6 ml. in the 1st hr. and 3·8 ml. in the 7th. The uptake of O_2 is very rapid for the 1st hr. and decreases slowly until the ratio CO_2/O_2 again approximates to unity. Evans drew attention to differences in the behaviour of fresh and withered leaf after crushing but was unable to offer any explanation. The increase in the oxidizing activity of the preparation of soluble enzyme which we have studied offers some explanation and the joint observations point to some vital changes in the respiratory processes of withered tea leaf which under Ceylon conditions is in a moribund condition, the withering process being carried beyond the permanent wilting point of the greater part of the leaf. The qualification with respect to the greater part of the leaf is necessary because we have observed surprising differences in the rate of wither below 65% moisture content even between pieces of flush gathered from the same bush [Lamb, 1936]. This finding is confirmed by Leniger & Deijs [1937] in Java.

Roberts [1939] has conducted an extensive manometric investigation of the fermentation of Assam tea leaf and most of his findings with *crushed fresh leaf* must apply to Ceylon leaf. Since, however, it is the practice in Ceylon to wither leaf much harder than in Assam some caution must be exercised in comparing results.

Discussion

Our study of the fermentation process has so far been analytical in its conception. The *in vivo* processes may be considerably more complicated than those in any of the component systems we have studied, but their eventual elucidation should be simplified by studies of the simpler component reactions.

It is of interest, however, to compare titrimetric estimations of activity *in vivo* with titrimetric estimations of activity *in vitro*. 0·1 g. of dry matter of freshly rolled leaf in the average case contains oxidizable substances equivalent to about 27 ml. $N/20$ $Na_2S_2O_3$. After 4 hr. fermentation this falls to the equivalent of about 21 ml. $N/20$ $Na_2S_2O_3$. In a typical *in vitro* experiment, an enzyme plus substrate system, roughly equivalent to 0·1 g. of dry matter of whole leaf, gave titrations of 26 and 20 ml. before and after a 4 hr. reaction period. The oxidizing activity observed in our reactions is therefore of the same order as the activity *in vivo* and there does not appear to be any doubt that our enzyme preparations contain the greater part of the polyphenol-oxidizing enzymes.

Undoubtedly, the enzyme preparation contains an oxidase system, which is independent of ascorbic acid, and there appears to be some doubt as to whether peroxidase activation takes place at all in the system we have studied.

The enzyme systems we have described have some properties in common with the several polyphenol oxidases but at present we will not confuse the

issues by the use of specific names or by conjectures about the parallels with plant respiratory systems which have been described by other workers. There is some evidence that the normal respiratory processes of tea leaf undergo vital changes during the withering process practised in Ceylon, and until such matters are further investigated reactions associated with respiratory changes must be examined with some care before they are considered in conjunction with the fermentation processes of Ceylon tea manufacture. It is conceivable, for instance, that the peroxidase and catalase systems of tea leaf function only in respiration and remain inactive during the fermentation process, except possibly in fragments of under-withered and comparatively undamaged leaf still respiring normally.

SUMMARY

An outline of the production of tea in Ceylon is given, and it is explained how enzyme oxidation of polyphenolic substances in the leaf is thought to be the principal change occurring during manufacture.

Methods are described for the preparation of enzyme practically free from polyphenols and a polyphenolic substrate free from active enzyme. A titrimetric method is employed for the observation of enzyme activity in the prepared substrate which is aerated during the course of the reaction.

The enzyme preparation may be resolved into a buffer-soluble part showing markedly increasing solubility from *p*H 5·0 to 8·0 and an insoluble part unaffected by successive leachings at any *p*H in the same range.

Oxidizing activity of the soluble enzyme in the prepared substrate is optimum at *p*H 6·0–7·0 and at 49°. The corresponding optima for the insoluble enzyme are *p*H 5·4 and 27°, the latter temperature being remarkably low for optimum enzyme activity of plant enzymes.

Both soluble and insoluble enzyme systems show marked specificity in oxidizing polyphenols with *o*-hydroxy groupings only. The primary oxidation products are most probably *o*-quinones but this has only been established by analyses in the case of catechol.

The addition of H_2O_2 to either the soluble or the insoluble enzyme systems causes increased activity but the system becomes less specific in action and will then oxidize *p*-dihydroxybenzene.

Enzymic absorption of O_2 accompanies the reaction between the enzyme preparations and the tea substrate, but CO_2 is not evolved when these enzyme systems oxidize tea substrate, although CO_2 is evolved during the *in vivo* fermentation processes.

The activity of the enzyme system studied is sufficient to account for all the polyphenol oxidation taking place during the fermentation process practised in the manufacture of tea in Ceylon.

2. OXIDIZING ENZYMES

ROBERTS & SARMA [1938] state that the fermentation of tea leaf in Assam is brought about by peroxidase and that oxidase is present only in negligible quantities. Roberts has latterly qualified this statement but Deijs [1939, 1, 2] has still more recently published findings to the effect that oxidase does not occur to any marked extent in Java leaf. Deijs used methods similar to those employed by Roberts and it is possible that, as with Roberts, the enzyme system capable of direct oxidation of tea tannins, which may exist in Java leaf, was also

inhibited by alcohol treatment at high laboratory temperatures, as reported in a previous communication [Lamb & Roberts, 1939].

The enzyme system which we have described readily oxidizes polyphenols without the addition of H_2O_2. In other words the system is an oxidase system. Our enzyme preparations are, however, more active in the presence of added H_2O_2 and the question arises as to whether the oxidase system contains any mechanism which may generate H_2O_2 to activate the peroxidase or whether the polyphenols are oxidized without peroxidase activation.

Experimental

Ascorbic acid oxidation

Roberts's [1939] first views were that H_2O_2 is generated from an ascorbic acid-ascorbic acid oxidase mechanism. Ascorbic acid is present in fresh Ceylon tea leaf in amounts varying from 0·2 to 0·4 mg. per g., but the specific ascorbic acid oxidase does not appear to occur in any of the Ceylon leaf we have examined. In our enzyme preparations, however, ascorbic acid is eliminated by the method of preparation, for leaching with acetone and, in the instance of the soluble enzyme, precipitation of buffer-soluble enzyme by means of alcohol, cannot leave any ascorbic acid associated with the enzyme preparation. In spite of this we have shown conclusively that the enzyme system will oxidize pure polyphenols without the addition of H_2O_2. If ascorbic acid oxidase were present in the enzyme preparation and H_2O_2 could be generated by oxidation of ascorbic acid, the addition of ascorbic acid to a reaction mixture of enzyme and polyphenol would be expected to cause acceleration of reaction. Careful experiments have completely failed to reveal any such acceleration. Roberts [1939] records similar observations. If ascorbic acid oxidase were present in fresh leaf, the mode of preparation of the enzyme powder with acetone would, according to Srinivasan [1936], be expected to preserve 33 % of the ascorbic acid oxidase activity of the powder. When ascorbic acid is added to an enzyme and catechol reaction mixture, the oxidation of the catechol, as shown by the formation of anilinoquinone in the presence of aniline, far from being accelerated is inhibited until the oxidation of ascorbic acid, followed by means of 2:6-dichlorophenol-indophenol, is complete. *o*-Quinones possess the power of oxidizing ascorbic acid and it has been shown that the rate of oxidation of ascorbic acid by our enzyme preparation is related to the production of quinones. Unfortunately none of our enzyme preparations has been completely free from polyphenols; as already mentioned, they all give a weak $FeCl_3$ test. However, the figures in Table 1 show that there is only a limited amount of quinone or potential quinone in the preparation and that the addition of polyphenol markedly accelerates the oxidation of ascorbic acid.

Table 1

Time min.	Control ascorbic acid solution	Enzyme from 0·05 g. powdered preparation (mg. ascorbic acid)			
		Soluble enzyme	Soluble enzyme + catechol	Insoluble enzyme	Insoluble enzyme + catechol
0	0·24	0·24	0·26	0·24	0·25
5	0·24	0·16	0·13	0·21	0·12
10	0·24	0·10	0·06	0·18	0·05
30	0·23	0·04	0·00	0·16	0·00

The acceleration of oxidation by catechol is less marked with the soluble enzyme which, as shown in Part I, Table 3, contains more of the polyphenolic residue after extraction by buffer than the insoluble enzyme preparations. Preparations of white amorphous tannin made from tea leaf have the same accelerating effect as catechol, thus further supporting the evidence for quinone formation from tea tannin.

KCN inhibition

Sensitivity to KCN is a well-known property of peroxidase. Evans [1927] stated that the uptake of O_2 by crushed (Ceylon) tea leaf is unaffected by the presence of HCN whilst Roberts & Sarma [1938] have stated that "small amounts of KCN completely inhibit fermentation" (in Assam). Accurate observation of the effect of addition of KCN to crushed leaf tissue is a difficult matter in practice because the intimacy of mixture is questionable, and because of possible interaction between the cyanide and substances not directly connected with enzyme activity. We have circumvented these difficulties by methods described in Part I and Table 2 gives the results of a detailed study of the inhibitory effect of KCN upon our enzyme preparations.

Table 2

	Concentration of KCN in reaction mixture. Enzyme activities expressed in ml. $N/20$ $Na_2S_2O_3$					
	0	$M \times 10^{-6}$	$M \times 10^{-5}$	$M \times 10^{-4}$	$M \times 10^{-3}$	$M \times 10^{-2}$
Enzyme powder + tea extract substrate	4·3	4·1	4·2	3·9	—	—
Enzyme powder + tea extract substrate + H_2O_2	6·3	5·8	4·6	3·4	—	—
Enzyme powder + purified amorphous tea tannin substrate	4·9	4·6	4·5	3·9	1·6	0
Enzyme powder + purified amorphous tea tannin substrate + H_2O_2	5·7	4·9	4·7	3·5	1·6	0

The conclusions to be drawn are quite clear and are that KCN up to a concentration of $M \times 10^{-4}$ has little effect upon oxidase activity while peroxidase activity stimulated by addition of H_2O_2 is markedly affected at $M \times 10^{-5}$. Oxidase activity is easily discernible at $M \times 10^{-3}$ concentration of KCN at which peroxidase stimulation is completely inhibited. Oxidase activity is inhibited at $M \times 10^{-2}$ concentration of KCN. These conclusions have been confirmed several times and appear to apply also to the soluble enzyme system, the peroxidase activity of which is inhibited at [KCN] $M \times 10^{-4}$ in catechol and gallic acid substrates while oxidase activity is still apparent. The dianilino *o*-quinone of catechol (M.P. 190°) was prepared by means of insoluble enzyme powder in the presence of $M \times 10^{-4}$ KCN.

Reviewing the data we have obtained we cannot avoid the conclusion that our enzyme preparation contains an oxidase system capable of oxidizing polyphenol without peroxidase activation. On searching the literature we have abstracted the following data concerning inhibition by KCN:

(*a*) Peroxidase activity stops at $5M \times 10^{-5}$ [Srinivasan, 1936].

(*b*) Ascorbic acid oxidase activity is very weak at $M \times 10^{-3}$ [Srinivasan, 1936].

(*c*) Cytochrome oxidase activity is inhibited at $1{\cdot}2M \times 10^{-4}$ [Stotz *et al.* 1938].

These data support our findings and cast doubt upon the presence of cytochrome oxidase in our preparations.

Non-fermenting leaf

During the course of plant selection work on tea, with which the Department is associated on account of selection for quality, we have come across a single bush of considerable biochemical interest. The flush from this bush does not ferment, however carefully the leaf is crushed and aerated. The following observations throw some light on the possible normal role of peroxidase:

(1) The tannin concentration is normal.

(2) The ascorbic acid content is normal.

(3) The addition of ascorbic acid and ascorbic acid oxidase does not induce any apparent change, either separately or in the form of lemon juice or apple juice.

(4) The addition of H_2O_2 induces fermentation as observed by colour changes of leaf and infused liquor.

(5) Preparations of enzymes from the leaf made by the methods previously described do not show any oxidase activity with our substrate preparation. Peroxidase action is stimulated by the addition of H_2O_2.

(6) Admixture of an active insoluble enzyme preparation (1·5 g. powder to 25 g. of leaf) induces a normal fermentation. In this case the colour of the infused leaf (tea-taster's "infusion") is more normal than in the case where H_2O_2 is added.

(7) Addition of Cu and Mn salts (*vide* Part I, § 6) does not induce any apparent change.

We admit that care must be exercised in applying conclusions drawn from abnormal specimens to normal cases, but we feel that the data obtained from the non-fermenting leaf support the evidence against the participation of ascorbic acid and ascorbic acid oxidase in normal fermentation. It also affords valuable indication that our enzyme preparation contains the essential part of the polyphenol oxidizing enzyme system of tea leaf.

Catechol oxidation

Oxidase and peroxidase activity in 0·2 % catechol solution in the presence of aniline both yield a dianilinoquinone, M.P. 191°. In the absence of aniline, peroxidase activity produces duller and more brownish products from catechol than oxidase activity, and it has been noted that whereas oxidase action produces very little change in I_2 titre, peroxidase produces a marked change in titre, the suggestion being that peroxidase activity results in the formation of more condensation products than does oxidase activity. This is probably true of other substrates and it appears to us that the addition of H_2O_2 to fermenting tea leaf results in duller and less desirable products from the tea-making point of view than does normal fermentation. Further investigation of this aspect of tea fermentation is desirable and may have important practical bearings.

The possible role of cytochrome

In a joint note [Lamb & Roberts, 1939] reasons were given why our Ceylon enzyme preparation might be expected to contain a cytochrome *c*-cytochrome oxidase system. Further investigations have led us away from the cytochrome theory though Roberts informs us of yet unpublished work in which he finds support for the idea that a cytochrome system exists in the insoluble enzyme preparation.

First, we have attempted to prepare cytochrome *c* by the method of Coolidge [1938]. We were able to follow this method without any difficulty and obtained a preparation similar to that described by the author but, in the absence of any

spectrometric apparatus, we were unable definitely to establish the presence of cytochrome *c*. However, the preparation was found to induce the oxidation of quinol and *p*-phenylenediamine by a washed beef muscle preparation (indophenol oxidase), which affords strong presumptive evidence of the presence of cytochrome *c* in our preparation. The addition of part of this preparation to our insoluble enzyme preparation did not accelerate the oxidation of tea extract substrate nor did it induce the oxidation of quinol or *p*-phenylenediamine.

Secondly, as mentioned in a previous section, our insoluble enzyme preparation retains some activity in a KCN concentration of $M \times 10^{-3}$, whereas Stotz *et al.* [1938] report inhibition of cytochrome oxidase activity at $1{\cdot}2\,M \times 10^{-4}$ concentration of KCN.

Thirdly, the insoluble enzyme does not oxidize quinol or *p*-phenylenediamine as a cytochrome *c* and cytochrome oxidase system would be expected to do. The insoluble enzyme powder, after several hours in a well aerated 0·1 % quinol solution at *p*H 5–5·5, shows no sign of oxidizing activity. 0·1 % *p*-phenylenediamine substrate under similar conditions shows some sign of oxidation in that the enzyme powder becomes stained with a blue colour, and after some considerable period the substrate itself becomes faintly blue to an extent slightly more pronounced than may be accounted for by autoxidation. Addition of catechol or white tea tannin preparation greatly accelerates the development of this blue colour. In the absence of added polyphenol the blue colour developed in the substrate is not extracted on shaking with toluene, the toluene layer remaining practically colourless. When catechol is added to the *p*-phenylenediamine substrate the development of blue colour is accelerated and a rose-coloured substance is extracted by toluene. If *p*-phenylenediamine be added to a catechol substrate + enzyme system which has been allowed to react for a time, the blue colour is developed instantaneously and toluene extraction results in a yellow colour in the toluene layer. Pure *p*-quinone rapidly causes the formation of the blue colour in *p*-phenylenediamine solution in a manner very similar to the enzyme plus catechol system. Since, as stated above, the insoluble enzyme powder contains adsorbed polyphenols it would appear that quinones and not direct enzyme activity are responsible for the slight observed oxidation of *p*-phenylenediamine. When α-naphthol is present together with *p*-phenylenediamine (Nadi reagent) similar observations have been recorded, but in this case toluene extraction results in a deep rose-coloured extract. Pure *p*-quinone produces instantaneous development of the blue-coloured substance soluble in toluene when added to Nadi reagent. Quinol in the presence of appreciable quantities of catechol or white amorphous tea tannin also appears to contribute to the development of a colour which cannot be accounted for by the oxidation of these latter polyphenols and there appears to be reason to suspect that quinol is also oxidized by the *o*-quinones of catechol and tea tannin although not so easily as *p*-phenylenediamine.

During the course of these investigations we found that a solution containing pure *p*-quinone and quinol slowly develops a red colour on the addition of H_2O_2, similar to that produced by tea peroxidase activity. The colour is not produced when either H_2O_2 or *p*-quinone alone is added to quinol solution. The possibility of quinone produced by oxidase giving apparent peroxidase activity in the presence of added H_2O_2 requires investigation.

A further characteristic of the system cytochrome oxidase plus cytochrome *c* appears from the literature to be its inhibition in reduced light intensity by CO, and the restoration of this activity in bright light. We have exposed crushed tea leaf, and enzyme preparation moistened with buffer, to an atmosphere of pure

CO for 30 min. under conditions of very low light intensity but have not been able to observe the slightest sign of inhibition in either case when compared with controls. The crushed leaf naturally does not ferment in an atmosphere of CO but fermentation commences immediately after exposure of the leaf to air. We have not observed the effect of mixtures of CO and air since, from the literature at our disposal, we gather that the inhibitory effect of CO upon cytochrome oxidation is permanent in low light intensities. The above investigations present a considerable bulk of evidence against the idea that the insoluble enzyme preparation contains cytochrome oxidase. However, we intend to make every endeavour to have a spectroscopic examination made on our enzyme preparation so that direct evidence for or against cytochrome *c* will be available.

Discussion

The ascorbic acid–ascorbic acid oxidase mechanism advanced by Roberts to explain the activation of peroxidase in tea leaf fails to account for the oxidizing activity of enzyme preparations made by us from Ceylon tea leaf. The oxidation of ascorbic acid added to fermenting tea leaf may be explained by the action of *o*-quinones upon ascorbic acid. Further work in Assam has led Roberts to modify his views and we have had the much appreciated opportunity of reading Roberts's unpublished work which has led him to abandon the theory.

The question therefore arises as to whether peroxidase activation takes place at all during fermentation or whether peroxidase is a respiratory enzyme which does not function during the degradation processes which constitute tea manufacture. We cannot at this stage obtain a complete answer to this question since we have only studied a part of the whole (*in vivo*) fermentation process, but the component which we have studied is sufficiently active to be able to account for all of the polyphenolic oxidation *in vivo*. The KCN-sensitivity of the oxidizing action of this component leaves little room for doubt about its independence of H_2O_2. Added H_2O_2 causes acceleration of oxidizing activity but this activity is rapidly reduced by KCN to the level of oxidizing activity in a system to which H_2O_2 has not been added. Furthermore, the system has been shown to possess the power of forming anilinoquinones in the presence of quantities of KCN which are known to inhibit peroxidase activity.

The specificity of the oxidizing activity with respect to *o*-dihydroxy groupings in polyphenolic substrates, pointed out in Part I of this series, also precludes peroxidase activation because on adding H_2O_2 the increased oxidizing activity is accompanied by loss of specificity, the enzyme systems then being capable of directly oxidizing polyphenols containing *p*-hydroxy groupings. The enzyme system in the presence of catechol or amorphous tea tannin, that is to say, when *o*-quinones are formed, appears to oxidize *p*-phenylenediamine and also quinol but this oxidation is in fact a secondary reaction due to *o*-quinones. Both the soluble and insoluble enzyme systems may, therefore, give a false impression of activity in the case of *p*-phenylenediamine, or Nadi reagent, and quinol, on account of *o*-quinone formation, whereas the enzyme systems free from any marked quantities of *o*-dihydric polyphenols are quite inactive. This appears to be a parallel to the ascorbic acid–ascorbic acid oxidase and the cytochrome c–cytochrome oxidase systems in other plants where a highly specific oxidase, through the medium of a substrate containing specific groupings, catalyses the initial changes of a series of reactions. Since tea leaf contains a much higher amount of polyphenolic substances than is usual in plant tissues, and is indeed cultivated for this reason, it would not be surprising if it contained also enzyme systems of rather specialized functions.

The literature on oxidizing enzymes contains many references to H_2O_2 formation as a result of the oxidation of dihydric phenols to quinones by polyphenolases. Pugh & Raper [1927] discuss this reaction in relation to "direct oxidases" which have a direct bearing on the problem of tea leaf oxidases. Our results do not lend any support to the suggestion that any H_2O_2, formed during the reaction between the enzyme systems we have studied and polyphenolic substrates, activates peroxidase. KCN-sensitivity and the specificity of these enzyme systems afford definite evidence to the contrary.

The conclusion we draw from our investigations is, therefore, that the soluble and insoluble enzyme systems are capable of direct oxidation of *o*-dihydric polyphenols without the activation of peroxidase, and are responsible for the primary activity in tea fermentation in Ceylon.

Summary

The enzyme systems described in the first paper of this series have been found to be comparatively insensitive to KCN, being active up to KCN concentration of $M \times 10^{-3}$, and completely inhibited only at $M \times 10^{-2}$. The specificity for *o*-dihydroxy groupings in polyphenols has been further studied and it has been shown that the oxidation products of catechol (*o*-quinone) and white amorphous tea tannin preparation (most probably *o*-quinones) oxidize ascorbic acid, *p*-phenylenediamine and, apparently, quinol. These secondary reactions may appear confusing in certain cases where the enzymes in the presence of tea polyphenols give positive reactions, as for instance in the case of Nadi reagent. During the oxidation of catechol and tea extract substrates *in vitro* the peroxidase present in the enzyme preparation is not activated. When H_2O_2 is added to the enzyme preparations there is a marked increase in activity but a loss of specificity. Quinol is rapidly oxidized when H_2O_2 is added to the system and attention is drawn to a possibility that *o*-quinones may, in conjunction with H_2O_2, form powerful oxidizing agents giving apparent peroxidase activity.

The primary reaction under *in vitro* conditions is the oxidation of *o*-dihydroxy groupings in polyphenolic substrates and the enzyme system responsible appears to be characteristic in that it does not conform to the description of any other well-known oxidizing enzyme system. It is possible that the oxidizing enzyme system of tea leaf, which has an unusually high polyphenol content, is a specialized system.

The authors wish to express their thanks to Dr R. V. Norris, Director of the Tea Research Institute of Ceylon, for permission to publish their work.

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176. ON THE SO-CALLED "IODIDE OXIDASE". MECHANISM OF IODIDE OXIDATION BY *ASPERGILLUS*

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(*Received 16 September 1940*)

It is well known that different organisms have the property of oxidizing iodides to free iodine. This reaction was first described by Schönbein [1848]. It was used by Bach & Chodat [1904] and among other workers by Wolff [1914; 1917] who suggested that the oxidation was due to a secondary reaction between the iodide and the oxidation products of polyphenols. Raciborski [1905], however, found that *Aspergillus niger* excreted an iodide-oxidizing system into its medium, but that both phenol oxidases and peroxidase were absent. Hence he postulated the existence of a specific iodide oxidase.

Korczewski [1922] confirmed Raciborski's observation and showed that the iodide-oxidizing system was destroyed by heat and acid and inhibited by KCN, NH_2OH and peroxide. Further, if the iodine is not removed an equilibrium is reached and the reaction soon comes to an end. He always found peroxide present in the medium, but, since its concentration was very low, he concluded that it could not be responsible for the oxidation of iodide. He found, however, that the addition of peroxidase caused a marked acceleration of iodide oxidation. Korczewski concluded that the culture medium contains an oxygenase similar to that postulated by Bach & Chodat [1902; 1903; 1904], and that this enzyme forms with molecular oxygen a superoxide which is responsible for the oxidation of iodide.

Methods

The methods used for preparing an active iodide-oxidizing medium were based on Korczewski's work. *A. niger* was grown on a medium composed of: 4 % sucrose, 0·2 % K_2HPO_4, 1 % $(NH_4)_2SO_4$, 0·05 % $MgSO_4,7H_2O$. It was found most convenient to buffer the medium to *p*H 7·8 with *M*/15 phosphate, inoculate it heavily with spores and incubate for 36–44 hr. at 30°; this usually gave a light continuous mycelium and the *p*H seldom fell below 4·5.

Before inoculation all media were sterilized by heating under pressure to 120° and allowing to cool rapidly. Cultivation was carried out in 500 ml. Erlenmeyer flasks containing 100 ml. of medium. The stock cultures were grown on wort agar slopes, and were subcultured once a month.

Manometric experiments were carried out at 39° in Barcroft manometers, all results being checked by control experiments. When any volatile or possibly autoxidizable substances (e.g. acetaldehyde) were used, the same quantity of these substances was placed in the compensating vessel as in the reaction vessel. The total volume of solution in each vessel was always 3·3 ml.

Factors involved in the oxidation of iodide

Six flasks of medium were prepared by Korczewski's method. The *p*H of the contents of each flask was determined and the iodide-oxidizing activity estimated. 10 ml. from each flask were pipetted into 100 ml. conical flasks which received

also 2 ml. of 0·4 % KI solution and two drops of starch. The free iodine formed was titrated with $1{\cdot}199 \times 10^{-2}$ *N* $Na_2S_2O_3$ at intervals during $2\frac{1}{2}$ days. Iodide oxidation appeared to proceed continuously, the rate of oxidation being greater with the lower *p*H. This was in agreement with Korczewski's results (see Table 1). The contents of all flasks gave also a positive reaction for H_2O_2 with benzidine and peroxidase but a negative reaction for peroxidase.

Table 1. *Effect of* p*H on the rate of oxidation of iodide by the culture medium of* Aspergillus niger

Oxidation expressed in ml. of $1{\cdot}199 \times 10^{-2}$ *N* $Na_2S_2O_3$

Time	*p*H 3·6	*p*H 3·8	*p*H 4·4	*p*H 4·6	*p*H 5·2	*p*H 6·8
70 min.	0·05	0·05	+	+	—	—
95 min.	0·20	0·20	0·07	0·10	0·03	+
20 hr.	0·80	0·85	0·42	0·45	0·18	0·10
24 hr.	0·90	0·95	0·47	0·50	0·18	0·10
43 hr.	1·25	1·10	0·62	0·62	0·25	0·14
45½ hr.	1·50	1·25	0·77	0·88	0·60	0·64
48 hr.	1·50	1·25	0·77	0·93	0·68	0·82
63 hr.	1·58	1·25	0·81	0·93	0·76	0·86

Since Korczewski had asserted that peroxide was always present in the active medium, it was thought advisable to check his two findings, namely: (1) that the oxidation of iodide is not due to peroxide, and (2) that it is inhibited by potassium cyanide.

For this purpose the culture medium was prepared as usual and the following quantitative estimations of the oxidation of iodide were carried out: (1) iodide oxidized by the media alone, (2) by media in the presence of *M*/1000 KCN, (3) media in the presence of *M*/100 KCN, (4) boiled media, (5) dialysed media and (6) the O_2 present as H_2O_2 was also determined.

For these estimations 10 ml. of each medium were used and 5 ml. of 0·4 % KI solution, except in the case of experiment (6), which was carried out in Barcroft manometers using 3 ml. of medium, 0·1 ml. of water and 0·2 ml. of catalase. The catalase was placed in hanging tubes which were dislodged after equilibration of temperature and closing of the taps.

The results of these experiments (Table 2) show that (1) the presence of *M*/1000 KCN accelerates iodide oxidation while *M*/100 KCN causes inhibition, and what is more important (2) the peroxide present in the medium is not

Table 2. *Iodide oxidation in 10 ml. of culture medium in 70 hr.*

Estimated in ml. of $1{\cdot}199 \times 10^{-2}$ *N* $Na_2S_2O_3$ and expressed as μl. of oxygen equivalent

Flasks	Untreated	$+10^{-3}$ *M* KCN	$+10^{-2}$ *M* KCN	H_2O_2 as μl. O_2
1	6·05	60·5	3·36	17·7
2	10·08	80·6	3·36	17·6
3	15·45	137·8	15·45	13·2
4	8·74	39·0	0·00	16·1

sufficient to account for all the iodide oxidation. The results with cyanide do not agree with those of previous workers, who, unfortunately, do not state what strength of cyanide was used, or how long the reaction mixture was allowed to stand. If they used cyanide at concentrations of about *M*/100, they could not expect any oxidation of the iodide to be apparent for some time, since any free iodine would immediately react according to the equation $KCN + I_2 = KI + ICN$, and not until all cyanide had been thus oxidized could any free iodine appear.

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That this is the explanation of the apparent inhibition obtained by previous workers is clearly demonstrated by further experiments in which the effects of cyanide and thiosulphate are compared.

In another series of experiments the estimation of peroxide and of oxidation of iodide were carried out in media: (1) untreated, (2) dialysed, (3) boiled and (4) mixture of boiled and dialysed after standing for 24 hr. The iodide oxidations were only allowed to proceed for 24 hr. The results of these experiments (Table 3)

Table 3. *Estimation of H_2O_2 and iodide oxidation*

H_2O_2 in μl. of O_2 and iodide oxidation in μl. of O_2 equivalent in 10 ml. of culture medium (untreated, dialysed, boiled and mixture of boiled and dialysed media). The peroxide in the mixture of boiled and dialysed media was determined after standing for 24 hr.

	Untreated		Dialysed		Boiled		Boiled and dialysed	
Flask	H_2O_2	Iodide	H_2O_2	Iodide	H_2O_2	Iodide	H_2O_2	Iodide
1	60·8	16·8	0	1·34	60·8	0	60·8	8·06
2	41·2	10·75	0	0	41·2	0	56·6	6·05
3	52·0	12·1	0	0	52·0	0	62·4	5·38
4	20·33	16·8	0	0	30·5	5·37	60·9	16·8
5	36·13	16·8	0	0	51·6	0	56·7	27·55
6	52·13	30·2	0	0	62·6	2·69	62·6	34·6

clearly show that at least two factors are involved in iodide oxidation: one is heat-labile and the other is dialysable. Further, the high peroxide concentrations in the mixtures of boiled and dialysed media indicate that there must be a source of peroxide in the medium. Thus, the fact that the iodide oxidation could exceed in its equivalent the amount of peroxide present in the medium before addition of iodide, could no longer exclude the participation of peroxide. This was ultimately confirmed by the experiments carried out in the presence and absence of catalase. For this purpose, four flasks of medium were prepared as before except that after sterilization and before inoculation 1 ml. of sterilized catalase was added to two of the flasks. The catalase was sterilized by precipitation with absolute alcohol, and redissolved in sterile distilled water. The following estimations were carried out on these media: (1) peroxide in 10 ml. of media, (2) iodide formed in 24 hr. in 10 ml. of media in the presence and absence of catalase and (3) in the presence of $10^{-3}M$ cyanide added to media devoid of catalase.

The results of these experiments (Table 4) show that catalase added either before or after incubation entirely destroys the iodide-oxidizing activity of the

Table 4

KI oxidation per 24 hr., and H_2O_2 concentration expressed as μl. O_2

	No catalase		Catalase	
Flask 10 ml. of media	1	2	3	4
H_2O_2	30·25	110·3	0·0	0·0
KI oxidation	11·4	13·4	0·0	0·0
KI oxidation in $10^{-3}M$ KCN	33·6	40·3	—	—
KI oxidation after catalase addition	0·0	0·0	—	—

medium. This clearly demonstrated that the oxidation is due entirely to the reaction: $H_2O_2 + 2HI = 2H_2O + I_2$.

The O_2 uptake by culture medium

Examination of samples of medium in Barcroft manometers reveals the presence of an oxidizing system with an O_2 uptake of about 45 μl. per hr. per ml. If all the O_2 absorbed were reduced to peroxide, the latter would be sufficient to account for the iodide oxidations already observed. The effect of KI on the O_2 uptake was examined and is shown in Fig. 1. The free iodine formed was titrated with thiosulphate and was found to vary directly as the square of the initial iodide concentration (Fig. 2) which forms additional evidence for the reaction $H_2O_2+2HI=2H_2O+I_2$, since H_2O_2 may well be kept at a fairly constant value by the enzyme system.

That the inhibition of O_2 uptake by KI in these experiments is due not to the iodide itself but to the free iodine formed was easily shown by the fact that the addition of $Na_2S_2O_3$ removes this inhibition (Fig. 3).

A more striking result was obtained in the presence of $M/170$ KCN instead of thiosulphate. The cyanide not only prevented the accumulation of free iodine, but by inhibiting all traces of catalase made the total peroxide formed in the medium available for the oxidation of iodide, with the result that the O_2 uptake was almost double that of the medium alone (Fig. 4).

The primary oxidation system in the medium and origin of H_2O_2

It can be definitely stated now that the oxidation of iodide in the culture medium is a secondary oxidation reaction due to H_2O_2 formed as a result of the activity of the primary oxidation system present in the medium. This is strongly supported by the results of experiments related in previous sections which can be summarized as follows:

(1) The oxidation of iodide in the culture medium depends on two kinds of factors, (*a*) thermolabile and non-dialysable—the enzyme, and (*b*) thermostable and dialysable—the substrate and H_2O_2 formed in the primary reaction.

(2) The medium alone has a distinct O_2 uptake, which shows that it contains a complete oxidizing system, namely the enzyme and its substrate.

(3) The H_2O_2 is gradually formed in the medium exposed to air, which shows that the O_2 in the primary oxidation reaction is reduced to peroxide.

(4) The iodide oxidation in the medium is inhibited by the addition of catalase which destroys the H_2O_2.

(5) The O_2 uptake by the medium is doubled on the addition of iodide and cyanide, the latter acting only as inhibitor of catalase present in the medium.

The nature of the primary oxidation reaction and the mechanism of iodide oxidation

The simplest way of determining the nature of the primary oxidizing system consists in separating the enzyme from its substrate and in identifying the latter. For this purpose the culture medium obtained in the usual way was dialysed for at least 16 hr. against running tap water and after dialysis precipitated with five volumes of acetone. The precipitate was centrifuged off and redissolved in 10 ml. $M/5$ acetate buffer pH 5·6. The activity of this preparation tested with boiled medium used as a substrate shows a yield of at least 80 %.

In order to identify the specific substrate which is oxidized by this enzyme preparation, its catalytic activity was tested with about 20 different substances such as succinic, lactic, malic and glycollic acids, alanine, glutamic acid, uric acid, alcohol, acetaldehyde, glucose, fructose, p-phenylenediamine and different

O_2 uptake in μl.
O_2 uptake in μl.

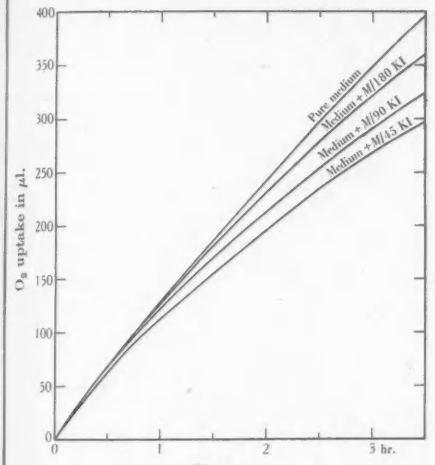

Fig. 1.

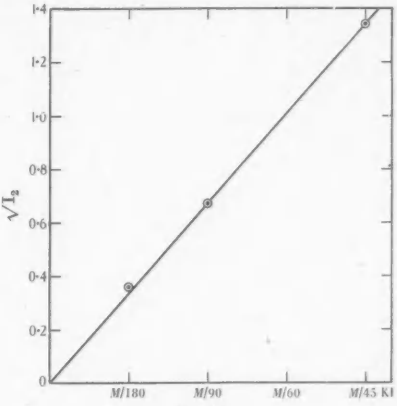

Fig. 2.

Fig. 1. The effect of iodide on the O_2 uptake of the medium.

Fig. 2. The relation between the concentration of iodide and the free iodine formed.

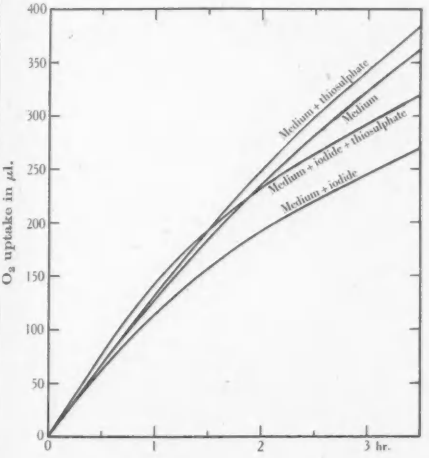

Fig. 3.

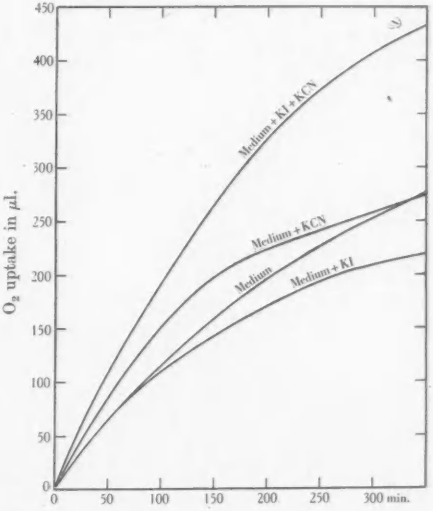

Fig. 4.

Fig. 3. The effect of iodide on the O_2 uptake of the medium in the presence of thiosulphate. 2·7 ml. medium; 0·4 ml. $M/4$ KI; 0·4 ml. $M/40$ $Na_2S_2O_3$.

Fig. 4. The effect of iodide on the O_2 uptake of the medium in the presence of cyanide.

phenols such as phenol, catechol, quinol, resorcinol, phloroglucinol. Of all these substances only glucose acted as a substrate for this enzyme preparation.

The enzyme responsible for the primary oxidation reaction in the medium can therefore be identified with the glucose oxidase of *A. niger* discovered and described by Müller [1928; 1929, 1, 2; 1931], reinvestigated by Franke & Lorenz [1937] and purified by Franke & Deffner [1939]. This view is strongly supported by further experimental results which can be summarized as follows:

(1) In absence of glucose the acetone preparation of the enzyme prepared from culture media has no O_2 uptake and does not oxidize iodide.

(2) In presence of $M/6$ glucose the preparation shows a marked O_2 uptake and iodide undergoes a secondary oxidation doubling the O_2 uptake of the enzyme-glucose system alone.

(3) This secondary oxidation of iodide is abolished on the addition of catalase.

(4) If in sucrose media the oxidation of iodide was due to the peroxide formed by the glucose oxidase system, it might be expected that in media having no source of glucose iodide would not undergo oxidation. Accordingly, the mould was cultivated on two fresh media, one containing galactose, the other fructose. After 68 and 44 hr. growth respectively, the media were filtered and to 5 ml. of each of them were added 50 mg. of KI. On allowing to stand for 48 hr. no oxidation of iodide was apparent. Further, when the media were examined manometrically the galactose medium showed no O_2 uptake and the fructose medium only a slight uptake of 0·7 μl. O_2 per ml. per hr.

(5) I_2 was found also to act as a hydrogen acceptor which was demonstrated by anaerobic experiments carried out in the usual way in Thunberg tubes. These experiments, summarized in Table 5, suggest that the decrease in the O_2 uptake

Table 5. *Reduction of I_2 by glucose oxidase ± glucose in absence of oxygen*

Thunberg tubes	Enzyme ml.	Acetate *p*H 5·6 ml.	$M/100$ I_2 ml.	$M/10$ glucose ml.	Water ml.	Time of decoloration min.
1	1·0	0·5	0·1	1·0	—	9–9·5
2	—	0·5	0·1	1·0	1·0	55–60
3	1·0	0·5	0·1	—	1·0	⩾60

by the system in presence of iodine is partly due to its competition with O_2 in acting as hydrogen acceptor. The reduction of iodine by this system explains also Korczewski's observation that during the oxidation of iodide to free iodine an equilibrium is reached which prevents the further accumulation of iodine in the medium.

(6) The enzyme system of the medium shares with the glucose oxidase and other oxidizing systems which reduce molecular oxygen to H_2O_2 the property of promoting secondary oxidation reactions. The addition of alcohol and catalase to such systems doubles their O_2 uptake. In this reaction, as was shown by Keilin & Hartree [1936], the oxidation of alcohol to aldehyde by H_2O_2 formed in the primary oxidation reaction is catalysed by small concentration of catalase. An increase in O_2 uptake by the media was also observed on addition of acetaldehyde which is oxidized to acetic acid. This secondary oxidation reaction, like that of iodide, is on the contrary inhibited by catalase. Using phosphate instead of acetate buffer, the formation of acetic acid can be easily demonstrated by the

lanthanum test. The secondary oxidation of the aldehyde was also followed manometrically and as shown in Fig. 5 the addition of acetaldehyde to the primary oxidation system doubles its O_2 uptake.

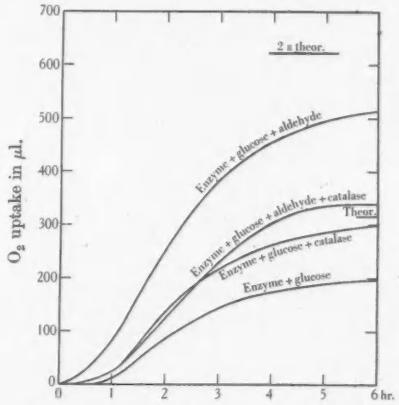

Fig. 5. The secondary oxidation of acetaldehyde by the glucose oxidase system in phosphate buffer. 5 mg. glucose; 30 mg. freshly prepared acetaldehyde.

(7) Finally the enzyme system of the medium, like Müller's glucose oxidase, is not inhibited by KCN, NaN_3, H_2S, NH_2OH or $Na_4P_2O_7$. On the contrary these reagents, as shown in Table 6, if anything accelerate the O_2 uptake of the system. On the other hand NaF was found to inhibit slightly and $NaHSO_3$ considerably the activity of this enzyme.

Table 6. *Effect of inhibitors on the O_2 uptake by culture media*

Expressed as % acceleration (+) or inhibition (−)

	%		%		%
M/250 NaN_3	+55	M/50 Na_2S	+86	M/50 NaF	−18·7
M/500 NaN_3	+47	M/50 $Na_4P_2O_7$	+11·1	M/100 NH_2OH	+23
M/1000 NaN_3	+29	M/50 $CH_2I.COONa$	+11·7	M/100 KCN	+28
		M/200 $NaHSO_3$ (pH 4·4)	−60		

All these experiments clearly demonstrate that there is no indication of the existence in the culture medium of *A. niger* of a special enzyme oxidizing iodide, as was postulated by Raciborski [1905] and Korczewski [1922]. It shows on the contrary that the oxidation of iodide in this medium is due solely to H_2O_2 formed in the primary oxidation of glucose catalysed by the glucose oxidase of Müller which diffuses out from the mycelium of the mould into the culture medium. Moreover, the secondary oxidation of iodide can be obtained in the presence of every primary oxidation system which like glucose oxidase or xanthine oxidase systems reduces molecular O_2 to H_2O_2, provided that the peroxide does not undergo destruction by catalase or by acting as hydrogen acceptor.

SUMMARY

1. The culture medium of *Aspergillus niger*, as was shown by previous workers, oxidizes iodide to free iodine.

2. This oxidation is not however catalysed by a specific iodide oxidase or by an oxygenase as was suggested by previous workers, but by hydrogen peroxide formed in the medium.

3. This peroxide is formed as a result of a primary oxidation reaction of glucose catalysed by glucose oxidase.

4. The oxidation of iodide is therefore a secondary oxidation reaction which can be promoted by every primary oxidizing system which reduces molecular oxygen to H_2O_2.

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177. THE CATAPHORETIC MOBILITY OF VISUAL PURPLE

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(*Received 11 June 1940*)

The photosensitive substance contained in the rods of the eyes of vertebrates, visual purple, is a chromoprotein, i.e. a protein to which one or more chromophores are attached. Aqueous solutions of visual purple are decomposed by light to give an unstable compound "transient orange", which in turn bleaches thermally to "indicator yellow" [Lythgoe, 1937]. Indicator yellow may also be formed without the action of light. Such treatment as is known to bring about denaturation of proteins is effective in bleaching visual purple and producing indicator yellow, e.g. heating of the solution, addition of alkali, acid or organic solvents and violent shaking. Wald [1937] found that a yellow pigment can be extracted with organic solvents from solutions of indicator yellow; this was explained by assuming that bleaching involves rupture of the bond between the protein and the chromophore.

The object of the present work was to determine the isoelectric point of visual purple and the change in its cataphoretic mobility on bleaching. A preliminary account of some of the experimental results has been given previously [Broda *et al.* 1939].

Experimental

To observe the mobility of visual purple a micro-electrophoretic method was chosen. The migration velocity of quartz particles (diameter 1–5 μ) suspended in buffered solutions of visual purple was measured under the microscope. Quartz particles suspended in protein solutions are coated with a layer of the protein; the sign of the charge and the mobility are within wide limits independent of size and shape of the particles, and are identical with those of the protein [Freundlich & Abramson, 1928; Abramson, 1928].

The cell consisted of a horizontal 64 mm. glass tube of elliptical cross section ($a_1 = 4{\cdot}9$ mm., a_2 (depth of the cell) $= 1{\cdot}6$ mm.) with glass stopcocks at both ends and a ground and polished centre part for observation. The electrodes were two platinized platinum wires introduced into the ends of the cell. The tension applied was 120 V., or 18·75 V./cm. Measurements were made at one-fifth of the depth of the cell. The values for the migration velocities observed at this depth were found to be very near to the absolute values, crystalline egg albumin serving as test substance.

By reversing the current the velocities of any single particle were determined in opposite directions in the cell and an average calculated in order to eliminate errors due to accidental flow in the solution. The final values for the velocities were obtained by taking an average of the velocities of a number of particles; but the individual variations of the different particles are normally not great. Examples for such series of observations are given in Table 1.

The visual purple solutions were prepared from the retinae of Hungarian frogs as described by Lythgoe [1937] and dialysed until practically salt-free. The concentration of the original solutions was of the order of 0·3 % chromoprotein, but after dilution with buffer it was reduced to about 0·01 %. In this range the mobility of visual purple and its bleaching product did not depend on concentration indicating that the quartz particles were well coated. The buffer used was $M/100$ sodium acetate-acetic acid; thus only uni-univalent salt was introduced. The pH was determined with a glass electrode in conjunction with a potentiometer set manufactured by the Cambridge Instrument Co., Ltd.

When the cataphoretic mobilities are plotted against pH, curves are obtained which, in the case of the compounds here investigated, closely approach straight lines at pH values where the mobility of the (positively or negatively charged) particles is small. The isoelectric point therefore is easily found by interpolation from the mobilities determined experimentally.

The experiments on visual purple were carried out in a safe red light. In each case after a sufficient number of observations, white light was switched on in the room and the velocity of the particles was measured again during and after bleaching of the solution. To exclude the remote possibility that different particles were involuntarily selected for observation by the eye in the red and white light respectively, in some cases the white light source was switched off after bleaching was complete, and the velocity in the bleached solution was measured again in red light. There was no difference between the velocities observed in the bleached solution with red or white light.

Results

At pH values below 5·1 a difference between the mobilities in the unbleached and bleached solutions was found. On illumination the velocities changed during a few minutes until a final value was reached; whether they increased or decreased depends on the particular pH value (sign of the charge). In the immediate neighbourhood of the isoelectric point the velocity change may amount to as much as 100 % and more.

As visual purple is known to be bleached by hydrogen ions the velocities obtained in unbleached solutions of comparatively high acidity cannot be taken to represent the true values. Actually no constant velocity is observed in solutions of $p\text{H} < 4{\cdot}2$ even in the dark, indicating appreciable bleaching of visual purple. In such solutions the velocity increases with time until a value is reached which is identical with that of the particles in an illuminated solution. The greater the acidity of the solution, the quicker the final value is reached. Table 1 shows by way of example three series of determinations at different pH values. At pH 5·11 the velocity does not change with time either in the dark or on illumination. At pH 4·26 the velocity changes on illumination, but not in the dark. At pH 3·85 the velocity changes not only on illumination, but a gradual change occurs in the dark. At pH values still lower the dark change proceeds so quickly that no reliable determination of the cataphoretic mobility of unbleached visual purple appears possible. It can be calculated from Lythgoe & Quilliam's [1938] data on the velocity of bleaching of visual purple by hydrogen ions that half of the substance will be bleached at 17° and pH 5·11 after about 300 hr., and at pH 4·26 after about 12 hr. These authors do not give the bleaching velocity at pH values as low as 3·85, but on extrapolation from their experiments it may be estimated that the half-life time of visual purple at this hydrogen ion concentration may be of the order of 10 min.

Table 1. *Three typical series of observations*

*p*H		Velocity (reciprocal values in arbitrary units)						
5·11	Dark:	− 3·45	3·25	3·20	3·40	3·25		
		3·20	3·45	3·75	3·50	3·15		
		3·30	3·30	3·45	3·45	3·20		
		Interval	3·10	3·15	3·45	3·40	3·30	3·30
			3·00	3·20	3·25	3·25	3·50	3·10
			3·05	3·20	3·35	3·35	3·40	3·20
	Illuminated:	3·00	3·35	3·20	3·45	3·30		
		3·70	3·30	3·40	3·20	3·60		
		3·35	3·30	3·30	3·30	3·45		
4·26	Dark:	+10·4	11·8	17·1	10·7			
		12·6	14·0	14·6	14·8			
		11·5	12·9	15·8	12·7			
	Illuminated:	7·5	7·4	7·6	6·5	5·8	7·2	
		7·9	7·6	7·2	8·3	8·0	7·8	
		7·7	7·5	7·4	7·4	6·9	7·5	
3·85	Dark:	+ 4·60	4·55	4·35	4·70	4·20		
		4·70	4·55	4·15	3·90	4·40		
		4·65	4·55	4·25	4·30	4·30		
		Interval	3·50	3·50	4·30	4·00	3·90	
			3·90	3·60	4·20	4·20	4·50	
			3·70	3·55	4·25	4·10	4·20	
		Interval	3·50	3·70	3·85	3·75	3·70	
			3·70	3·70	3·90	4·05	4·05	
			3·60	3·70	3·85	3·90	3·85	
	Illuminated:	3·60	2·90	2·90	3·10	2·85		
		3·15	2·70	3·00	3·10	3·10		
		3·40	2·80	2·95	3·10	3·00		
		Interval	2·90	2·50	2·90	2·90	2·90	
			3·20	2·65	2·95	2·45	2·90	
			3·05	2·60	2·90	2·70	2·90	

Notes. The upper and lower figures in the same column refer to the velocities in opposite directions obtained by reversing the current.

The time intervals are about 5 min.

The values at *p*H 4·26 (dark) scatter considerably, as the velocity is so small that water currents influence the result.

The results obtained with a particularly pure preparation of visual purple are shown in Fig. 1. According to these the most probable values of the isoelectric points of visual purple and its bleaching product are *p*H 4·47 and 4·57. In agreement with this a solubility minimum of the bleaching product was found between *p*H 4 and 5. The isoelectric point being far on the acid side of the neutral point the compounds are to be ranged among the so-called acid proteins. This explains the earlier observation [Lythgoe & Quilliam, 1938] that buffered solutions of visual purple tend to become acid on prolonged dialysis.

While the shift on illumination is observed in every experiment, the absolute values of the cataphoretic velocities of the individual samples show slight variations. These could be due (1) to partial denaturation during preparation or to the presence of varying amounts of (2) non-protein or (3) protein impurities. (1) Denaturation cannot occur to any marked extent. While visual purple invariably gives indicator yellow on denaturation, pure visual purple solutions do not show indicator properties. (2) Phospholipins are present in the solutions

[Krause, 1937; Broda, Goodeve & Lythgoe, unpublished]. It was found, however, that addition of large amounts of lecithin to a solution of a test substance (gelatin) does not influence its cataphoretic behaviour. Furthermore, the solutions, from their preparation, contained varying amounts of digitonin. This could be removed quantitatively from the solution by a short shaking with a solution of cholesterol in light petroleum; but in practice this treatment was

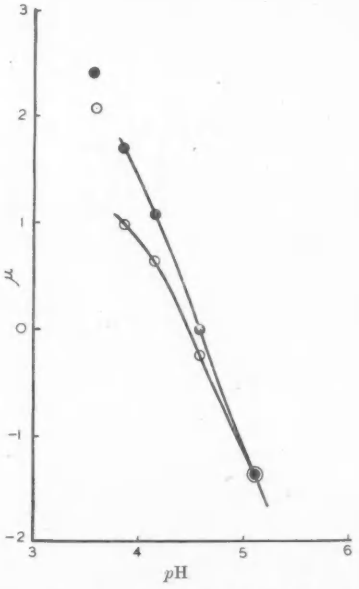

Fig. 1. The cataphoretic mobility of visual purple and its bleaching product. Open circles: visual purple; full circles: bleaching product.

unnecessary, as addition of much digitonin to a gelatin solution did not change the mobility of the particles. (3) Washing of the rod tissue with buffer of *p*H 4·5, which is a normal step in the preparation of the solutions, removes small amounts of a colourless protein. This is detected by its ultraviolet absorption band which disappears on addition of alcohol to the buffer solution. Part of this protein may conceivably remain in the tissue and be dissolved together with the visual purple when digitonin acts on the rods.

Discussion

The chemical significance of the charge difference. It is well known that intense irradiation of proteins, especially with ultraviolet light, may cause denaturation or far-reaching decomposition obviously leading to changes in their electrical properties. But apparently no protein has been known to undergo a shift in cataphoretic mobility on moderate illumination. This unique electrochemical behaviour of visual purple is connected with its high photochemical sensitivity as expressed in terms of light absorption.

The difference of the electric charges before and after bleaching must be due to a difference in the ionized groups in the adsorbed protein layer. The production of a similar electric charge difference in haemoglobin through chemical reaction has been observed by Hastings *et al.* [1924] and by German & Wyman [1937]; the titration curve of haemoglobin differs from that of oxyhaemoglobin, the oxidized compound being a stronger acid between *p*H 6·1 and 9·0 and a stronger base between *p*H 4·5 and 6·1. In view of Wald's assumption that bleaching of visual purple involves rupture of the bond between the protein and the chromophore, it is interesting that the isoelectric point of globin, freed from the chromophore haem by acids, is 0·7 *p*H unit higher than that of the chromoprotein haemoglobin [Roche, 1929].

The greater positive charge of visual purple after bleaching may be due to a decrease in the number of ionized acid groups as well as to an increase in the number of ionized alkaline groups. The first might be caused by a reduction of the strength of acid groups present (but not by the total suppression of one of them, as then the charge difference would remain unaltered at higher *p*H). The second may be brought about by a strengthening of alkaline groups present, or even by a liberation of new alkaline groups. Unfortunately, the instability of visual purple in acid solutions prevents a decision whether the charge difference is maintained at low *p*H (indicating that new alkaline groups are liberated on bleaching), or whether, as in the system haemoglobin-oxyhaemoglobin, the two curves unite at low as well as at high *p*H (indicating that only a modification in the strength of existing groups has occurred, and that the total number of dissociable groups has not changed). In the latter case the dissociation region is merely shifted on transformation, and the mobility or titration curves, even if they are extended over the widest *p*H range, cannot provide a criterion whether the groups transformed are acid or alkaline.

The dissociation constant of the group affected can be roughly estimated from the relationship between the charge difference and *p*H from 5·1 to 3·85, if one univalent group is assumed to be responsible. From the curvature of this function it follows that the midpoint of the dissociation region is somewhat below *p*H 4, and that the dissociation constant is therefore somewhat greater than 10^{-4}. This refers to the group in the bleached compound if the group is alkaline, and to the group in visual purple if the group is acid.

Dissociation constants as high as 10^{-4} are so far unknown among the basic groups of protein constituents, but are common among their acid groups. In the living retina (*p*H$\sim$6·5) the dissociation of the group will be practically complete.

The group apparently has nothing to do with the indicator properties of the bleaching product. The transformation region of indicator yellow is above *p*H 5 [Lythgoe, 1937].

The numerical value of the charge difference. The electric charge Q of a protein molecule at any *p*H value can be estimated by means of Abramson's [1932] equation

$$Q = 6\pi\eta v r\,([r\sqrt{m}\cdot 0{\cdot}33\times 10^{8}]+1)$$

(η = viscosity of the solution in poises, r = radius of the molecule, m = ionic strength of the solution, v = cataphoretic mobility in cm./sec./electrostatic units/cm.) As pointed out above, the mobility of the protein is identical with that of the coated quartz particle. Accordingly the charge difference ΔQ produced by bleaching visual purple at *p*H 3·85, i.e. the maximum charge difference which can be measured fairly safely, can be calculated from the mobility difference of 0·71 μ/sec./V./cm., if the protein is assumed to be spherical, and its

molecular weight and specific gravity are 270,000 [Hecht & Pickels, 1938] and 1·3, respectively:

$$\Delta Q = 6\pi . 0{\cdot}01 . 37{\cdot}3 \times 10^{-8} . 213 \times 10^{-4} \left([37{\cdot}3 \times 10^{-8} \sqrt{(0{\cdot}01)} . 0{\cdot}33 \times 10^{8}] + 1\right)$$
$$= 33{\cdot}5 \times 10^{-10} \sim 7 \text{ elementary charges.}$$

The dissociation constant of the group concerned being a little greater than 10^{-4}, the charge difference at *p*H 3·85 is due to not more than about half ionization of the group; should a modification in the strength of one of the existing groups be the reason of the charge difference, this may correspond to less than half ionization at the midpoint of the dissociation region, as then the ionization of the original, non-modified group has to be taken into account. Thus at full ionization not less than 14 elementary charges per visual purple molecule, or not less than one charge per weight of 19,300, would be obtained on bleaching. Similarly, on oxidation of haemoglobin (mol. wt. 66,800) at least three additional charges per molecule, or one charge per weight of 22,300, are produced [Hastings *et al.* 1924; German & Wyman, 1937]. The charge difference of not less than 14 compares with a number of 16 Svedberg units in the molecule of the visual purple protein, probably each unit carrying one chromophore [Broda *et al.* 1940]; it may be that light or hydrogen ions transform one group in each Svedberg unit, while bleaching the chromophore attached to it.

Summary

The cataphoretic mobilities of visual purple and its bleaching product have been determined between *p*H 3·5 and 5·1 and found to differ. The most probable value of the isoelectric point of visual purple is *p*H 4·47 and that of the bleaching product 4·57.

The shift in the mobility is due to a change in the ionized (acid or alkaline) groups in the protein molecule on bleaching. The nature of this change is discussed. The dissociation constant of the group concerned is of the order 10^{-4}.

The charge difference at the midpoint of the dissociation region of the group amounts to about 7 elementary charges per molecule of the chromoprotein. The molecules contain at least as many groups capable of transformation as chromophores.

Our sincere thanks are due to Dr C. F. Goodeve and the late Dr R. J. Lythgoe for enabling us to carry out this work, to them and to Dr G. S. Hartley for useful discussions, to Miss S. J. Hooper for the preparation of the visual purple and to the Rockefeller Foundation for a financial grant to one of us (E.E.B.).

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178. THE RESPIRATION AND ANAEROBIC FERMENTATION OF TEA LEAF AND THEIR RELATIONSHIP TO TEA FERMENTATION

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(*Received 10 October 1940*)

Comparatively little work has been carried out on the respiration of leaf tissue using manometric methods. Kempner [1936] measured rates of respiration of whole leaves from pine, tobacco, plum and oleander, in this manner, and Allen & Goddard [1938] recorded respiration rates of strips of leaf from wheat floated in water, using the Fenn micro-respirometer. Later, Marsh & Goddard [1939, 1], using this same apparatus, investigated the respiration of carrot leaf with 100 mg. samples floated on phosphate buffer. The success of these workers encouraged us to make similar observations on the rate of respiration of tea leaf.

These investigations into the respiration and anaerobic fermentation[1] of tea leaf, obtained both by the manometric and by the standard Pettenkofer methods, together with manometric measurements of the rate of tea fermentation and of simpler enzyme-substrate systems have enabled us to draw fairly definite conclusions as to the presence of the cytochrome system in tea leaf, and the almost complete inactivation of the H-carrying coenzymes (cozymase) on extensive mechanical damage to the leaf.

Methods

(1) *Manometric method of determination of respiration rate.* Disks, $\frac{1}{2}$ in. in diameter, are punched in the centres of the second leaf of shoots consisting of two leaves and a bud. 10–15 such disks, weighing approximately 250–400 mg., constitute a fairly representative sample; in fact in only one experiment out of 20 was there any serious variation between replicates.

The disks were suspended in 3 ml. water or $M/15$ phosphate buffer *p*H 5·8, contained in Warburg vessels. KOH, where necessary, was contained in the inner cups, rolls of filter paper being employed to ensure an adequate surface of the CO_2 absorbent. CO_2 outputs were obtained by difference between the mean uptakes of four vessels with KOH, and of another four without KOH, using the appropriate constants of the vessels for O_2 and CO_2. For plant tissues in a medium of water this method appears to give results quite as accurate as those obtained by the method of Dickens & Šimer [Turner, 1938].

In these determinations the Warburg apparatus was covered by a black cloth, thus preventing any disturbance of the O_2-CO_2 interchanges by photosynthetic processes.

[1] A careful distinction must be drawn between the two uses of the word fermentation. Tea fermentation is not an anaerobic process, and to avoid confusion is always referred to as tea fermentation.

(2) *Pettenkofer method.* 50–100 g. of leaf, fresh, withered or mechanically damaged, were placed in a respiration chamber. The gases (N_2 and air) were driven through this chamber from cylinders or aspirators at about 3 l./hr., first passing through two tall soda-lime absorption towers. A small $CaCl_2$ tube was placed between each respiration chamber and the Pettenkofer tubes, which were protected from the atmosphere by soda-lime tubes. At the end of each hour the excess $Ba(OH)_2$ was determined by addition of excess oxalic acid followed by back-titration with $Ba(OH)_2$. The results were expressed in ml. CO_2 absorbed.

In anaerobic experiments the respiration chamber containing the leaf was evacuated and filled with N_2 several times before absorption of CO_2 was started. This served to remove, to a large extent, CO_2 held by the tissue. No time could be allowed for the tissue to accommodate itself to fresh conditions as the withered and mechanically rolled samples of leaf are not in a condition where they are likely to survive for long. The values obtained are therefore probably not comparable with results obtained for leaf samples under more refined conditions but it is considered that this method, although not giving absolute values for anaerobic fermentation, will be sufficiently accurate to reveal differences in anaerobic fermentation due to mechanical damage to the leaf.

(3) *Tea fermentation.* Rates of tea fermentation were determined by the standard method described in earlier communications from this laboratory [Roberts, 1939; 1940].

Respiration of tea leaf

Results are most conveniently recorded as μl. gas per 100 mg. tissue, for successive half-hour periods. Table 1 shows a typical result.

Table 1

	Hours											
	0–½	½–1	1–1½	1½–2	2–2½	2½–3	3–3½	3½–4	4–4½	4½–5	5–5½	5½–6
O_2 uptake (μl.)	40	34	36	29	29	21	24	25	25	25	24	23
CO_2 output (μl.)	51	33	35	26	25	18	23	18	20	21	15	17
R.Q.	1·27	0·97	0·97	0·90	0·86	0·86	0·96	0·72	0·80	0·84	0·63	0·74

The rapid fall in respiratory activity in the first 2 hr. is not considered to be of any physiological significance; the tissue is probably adjusting itself to its new environment. The fall in the rate is certainly not due to carbohydrate depletion since in 6 hr. the total output of CO_2 is 302 μl. which is equivalent to 0·4 mg. glucose. 100 mg. of leaf contain more than this quantity of free glucose besides reserves of starch and other polysaccharides. The fall in the rate for the first 2 hr. therefore must be due to some other cause than substrate shortage.

The high initial R.Q., lasting as it does for only 30 min., is thought to be due to the expulsion of CO_2 from the intracellular spaces of the leaf as a result of the shaking at the higher temperature (36°). The preliminary equilibration seems to have been insufficient to have driven off this free CO_2 from the leaf.

There is a steady decrease in R.Q. throughout the whole period of shaking. Irregularities in this decrease are due to experimental error and taking all determinations into account no fluctuations such as occur in the above example are observed. It should be remembered that quite small errors in the original manometric reading may lead to an error of as much as 10% in the R.Q. This steady decrease in R.Q. cannot be put down to an increased share by protein and fat in respiration, as carbohydrate supplies in the leaf are ample for a considerably longer period of shaking than 6 hr. Some browning is observed at the

edges of the disks and we ascribe the decrease in R.Q. to a local "tea fermentation" brought about by disintegration of the tissue, near the cells damaged by the cut.

In view of these non-physiological factors, results on respiration obtained manometrically must be interpreted with caution; nevertheless the technique has proved quite useful for comparative purposes.

Effect of carbohydrate depletion on respiration. Leaf may be largely depleted of its carbohydrates by storage for several days in the dark, without appreciable change in turgor of the leaf if the atmosphere is saturated with water vapour. After 72 hr. a sour smell has usually developed, the leaf has a high bacterial infection, and a considerable proportion of the leaf surface will have browned. No enhancement in the rate of O_2 uptake as a result of this bacterial contamination has been observed.

The effect of this impoverishment of carbohydrates is to reduce both respiration rate and R.Q. Measurements of the respiration of leaf when fresh and after 1, 2 and 3 days' storage are recorded in Table 2. The figures represent μl./100 mg. tissue.

Table 2

	Days storage	Hours 0–½	½–1	1–1½	1½–2	2–2½	2½–3	3–3½	3½–4	4–4½	4½–5
O_2 uptake (μl.)	0	54	44	35	39	36	32	30	28	30	20
	1	50	44	34	30	31	29	32	24	22	20
	2	42	36	26	26	26	17	21	20	22	20
	3	44	32	24	28	24	24	24	20	23	23
CO_2 output (μl.)	0	60	47	37	39	36	33	28	27	27	18
	1	52	45	32	28	31	28	29	22	20	17
	2	40	33	24	23	23	13	17	18	18	18
	3	39	29	23	25	23	21	21	20	18	23
R.Q.	0	1·11	1·07	1·06	1·00	1·00	1·03	0·90	0·96	0·90	0·90
	1	1·04	1·02	0·94	0·93	1·00	0·96	0·90	0·92	0·91	0·85
	2	0·95	0·92	0·92	0·88	0·88	0·77	0·80	0·90	0·82	0·90
	3	0·98	0·91	0·95	0·89	0·95	0·87	0·87	1·00	0·78	1·00

The reductions in respiration rate and R.Q. during the first 2 days are significant. On the 3rd day there is no further fall in respiration rate, and a significant increase in the R.Q.

The results for the first 48 hr. suggest that as the carbohydrate content falls the rate of respiration falls, and that the proteins play an increasingly important part as respiratory material. The significant fall in the R.Q. is strong evidence in favour of the view that protein is being oxidized.

The check in the rate of fall of respiratory activity and the significant rise in the R.Q. are not immediately explicable although it is interesting to point out that Yemm [1935] found similar decreases for starving barley leaves, and that after 3 days the R.Q. reached a minimum value, rising again later. Both for barley and for tea this change is associated with marked browning of the leaves and it is possible that, as with other senescent plant organs, the "climacteric" is reached [Kidd, 1935] which is associated with a temporary rise in respiratory activity.

Tea fermentation is not affected by depletion of carbohydrates to the same extent as is respiration. Rates of tea fermentation determined on successive days were as follows:

Days starvation	$-Q_{O_2}$
0	19·2
1	18·2
2	17·1
3	20·8

Two important conclusions can be drawn from this result: (1) Losses of carbohydrates during the withering process in tea manufacture are unlikely to affect the rate of fermentation. (2) The oxidase activity is but little affected by 3 days' starvation; the fall in respiratory activity is therefore probably due to substrate shortage.

Inhibition of respiration and tea fermentation by HCN and CO

In the determination of the inhibitory effect of cyanide on respiration and tea fermentation, certain modifications in the manometric technique are necessary. The disks or minces are suspended in $M/15$ phosphate buffer pH 5·8, and the KOH is replaced by the KOH-KCN mixtures prescribed by Krebs [1935]. If KOH be used for CO_2 absorption when cyanide is present, the HCN is slowly absorbed by the alkali and the original inhibition is reversed. The reversibility of the cyanide inhibition of tea respiration may be demonstrated in this way.

It is difficult to overcome the effect of HCN absorption completely in this way and partial reversals of cyanide inhibition after 2 hr. shaking are frequently observed. This is attributed to slight errors in making up the KOH-KCN mixtures, and to variations in the HCN partial pressure of such mixtures caused by CO_2 absorption.

Typical effects of cyanide on the respiration of the 1st and 5th leaves of the growing shoot, and on tea fermentation are shown in Tables 3–5.

Table 3. *Inhibition by cyanide of the respiration of the 1st leaf in the growing shoot*

Molar conc. of cyanide	Min. 10	20	30	45	60	90	120	150
	μl. O_2/100 mg. tissue							
—	13	31	46	71	89	126	165	200
5×10^{-4}	9	19	28	41	44	55	60	70
2×10^{-3}	8	16	20	29	33	39	47	54
1×10^{-2}	7	14	20	26	29	35	43	45

Table 4. *Inhibition by cyanide of the respiration of the 5th leaf of the growing shoot*

Molar conc. of cyanide	Min. 10	20	30	45	60	90	120	150
	μl. O_2/100 mg. tissue							
—	14	24	34	42	54	74	94	111
5×10^{-4}	11	22	28	36	48	62	70	82
2×10^{-3}	10	19	26	34	42	50	57	63
1×10^{-2}	7	13	18	22	26	33	36	40

Table 5. *Inhibition by cyanide of tea fermentation*

Molar conc. of cyanide	Min. 10	20	30	40	50	60
	μl. O_2/100 mg. tissue					
—	75	128	170	184	193	199
5×10^{-4}	38	70	102	130	156	176
2×10^{-3}	22	36	50	62	76	90
1×10^{-2}	15	20	21	21	22	23

The cyanide apparently does not exert its full inhibitory effect on respiration for some 45 min. Table 6 shows the percentage inhibitions of the respiration as found in one experiment for the first 45 min. and subsequently.

Table 6. *Percentage inhibition of respiration by cyanide*

Molar conc. of cyanide	1st leaf of growing shoot		5th leaf of growing shoot	
	0–45 min.	45–150 min.	0–45 min.	45–150 min.
5×10^{-4}	43%	78%	14%	33%
2×10^{-3}	60	80	19	58
1×10^{-2}	64	86	48	74

For tea fermentation, initial rates show that 5×10^{-4} *M* HCN causes 49% inhibition and 2×10^{-3} *M* HCN 71% inhibition. The initial inhibition by 10^{-2} *M* HCN is still higher and after 20 min. this inhibition is almost complete.

The increased resistance of maturer leaf to cyanide poisoning is of interest, and recalls a similar finding by Marsh & Goddard [1939, 1] for carrot leaf. The consistent increase in the percentage inhibition with HCN concentration up to 10^{-2} *M* is also noteworthy. This is particularly marked in the case of tea fermentation.

Similar results are obtained with tea oxidase preparations acting upon pyrogallol and *p*-phenylenediamine as substrates.

Table 7. *Inhibition by cyanide of the oxidation of pyrogallol by tea oxidase*

Molar conc. of cyanide	Min.					
	10	20	30	40	50	60
	μl. O_2/100 mg. tissue					
—	108	190	252	312	366	404
5×10^{-4}	48	88	128	171	206	246
2×10^{-3}	23	33	49	62	72	98
1×10^{-2}	10	(8)	14	18	18	22

Table 8. *Inhibition by cyanide of the oxidation of* p*-phenylenediamine by tea oxidase*

Molar conc. of cyanide	Min.					
	10	20	30	40	50	60
	μl. O_2/100 mg. tissue					
—	37	58	77	91	112	118
5×10^{-4}	28	49	67	86	93	106
2×10^{-3}	18	37	51	64	70	80
1×10^{-2}	8	15	17	17	17	17

The comparatively low sensitivity of these mixtures of enzyme and substrate to cyanide inhibition is apparent, 10^{-2} *M* HCN being required for complete inhibition. From these results it appears unlikely that the low sensitivity of tea fermentation to cyanide is due to combination of the HCN with tannin or other HCN-binding substances in the system. It would seem that the tea oxidase requires as high a concentration as 10^{-2} *M* before its activity is completely inhibited.

In the above experiments figures are quoted for the rates of oxidation of pyrogallol and *p*-phenylenediamine by a tea-oxidase preparation. Ascorbic acid and quinol were also found to act as substrates for this enzyme. 1 ml. of substrate solution (100 mg./50 ml. water) was added to 1 ml. of a suspension of 100 mg. tea oxidase in 10 ml. *p*H 5·8, *M*/5 phosphate and rates of uptake measured manometrically.

Table 9. *Oxidation of quinol and ascorbic acid by tea oxidase*

	Min.			
	15	30	45	60
		μl. O_2/100 mg. tissue		
Ascorbic acid	26	44	59	76
Quinol	41	70	94	119

The relative rates of oxidation of various substrates by the tea oxidase are summarized below, expressed in terms of μl. O_2/hr./mg. of enzyme.

Table 10

Substrate	$-Q_{O_2}$
Ascorbic acid	10·4
Quinol	16·4
p-Phenylenediamine	22·2
Pyrogallol	65·1
Tea tannin	120*

* This value was obtained for a different sample of enzyme. Independent measurements confirm that tea tannin is oxidized about twice as rapidly as pyrogallol by the tea oxidase.

Inhibition by CO

An investigation into the reversal by light of the inhibition by CO of plant respiration is complicated by other effects of light on the interchange between O_2 and CO_2 in respiring plant tissue. Kempner [1936] overcame this by slight narcosis with $CHCl_3$ which he claimed inhibited CO_2 assimilation without decreasing the rate of respiration. With tea leaf such treatment results in a browning of the leaf due to tannin oxidation.

It was considered that the simplest way of investigating the effect of CO on the tea oxidase would be to use the method of Keilin [1929]. Tea oxidase preparations were incubated with the Nadi reagent in Thunberg tubes filled with various gas mixtures. At the end of 10 min. shaking the reaction was stopped by addition of 1 ml. 1 % KCN and the indophenol blue extracted with toluene and estimated colorimetrically. The effects of 75 % CO on the tea oxidase in the light and in complete darkness are summarized in Table 11.

Table 11

O_2 %	N_2 %	CO %	Illumination	Reaction
5	95	Nil	Light	+ + + +
5	20	75	Light	+ + +(+)
5	20	75	Dark	+ +

There was about 50 % inhibition when the reaction was carried out in the dark, but in the light inhibition did not exceed 10 %.

Anaerobic fermentation of tea leaf

Recent work by Marsh & Goddard [1939, 2] on the relation between respiration and anaerobic fermentation in carrot root tissue has shown that inhibition of respiration, either by lowered O_2 tension or by poisons such as HCN, results in increased fermentation and hence in a rise in the R.Q. The R.Q. of respiring tea leaf is similarly enhanced by cyanide inhibition.

Table 12. *Effect of cyanide on* R.Q. *of respiring tea leaf*

Molar conc. of HCN	μl. per 100 mg. leaf per hr. O_2	CO_2	R.Q.
Nil	120	144	1·20
2×10^{-3}	78	132	1·69
1×10^{-2}	42	84	2·00

The figures for O_2 uptake and CO_2 outputs are calculated from initial rates; hence the high R.Q. recorded for uninhibited respiration.

It was found possible to demonstrate a net evolution of CO_2 from tea leaf when disks were suspended in Warburg vessels in an atmosphere of N_2. The anaerobic fermentation however is better followed by the Pettenkofer method.

In one series of experiments, using 50 g. samples of fresh green leaf, the CO_2 evolved per hr. averaged 8·2 ml. (at N.T.P.) in a current of air, and 4·3 ml. in a current of N_2. As the quotient, CO_2 of fermentation/CO_2 of respiration, is greater than one-third, and in one experiment amounted to 0·69, the Pasteur effect is probably in operation. This cannot be established definitely unless it is certain that the ratio C_2H_5OH/CO_2 is close to unity in accordance with the equation for alcoholic fermentation,

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2,$$

and at the moment we have no evidence in this direction.

Effect of mechanical damage on anaerobic fermentation

In the previous section it was shown that inhibition of the respiration of tea leaf by cyanide caused an increase in the R.Q. No such increase could be found when the O_2 uptake of a finely minced tea-leaf suspension was inhibited by 10^{-2} *M* HCN. The total O_2 uptake of the poisoned system amounted only to 6 μl. after 1 hr. and CO_2 evolution was too small to be measured. The net changes were too small to be accurately measured but the R.Q. was certainly less than 0·2. Thus, in contradistinction to undamaged tea leaf, no anaerobic glucose breakdown is evident in the finely minced tissue.

This is shown even more strikingly by results obtained with a suspension of finely minced tea leaf in water, shaken in an atmosphere of N_2. Naturally, the O_2 uptake under anaerobic conditions is nil; in addition, the CO_2 output does not differ significantly from zero after 90 min. shaking. This is in marked contrast to the findings for the whole tea leaf. The mincing of the tissue has apparently destroyed the power of the tissue to undergo anaerobic fermentation.

The manometric method is not well adapted to measuring very small rates of CO_2 production, and these findings do not necessarily prove that the destruction of the ability to undergo anaerobic fermentation is complete. In a fine mince however it may be concluded that the rate of anaerobic fermentation does not exceed 5% of the rate in undamaged tissue.

The Pettenkofer method, where CO_2 is determined directly, is a more accurate means of estimating small quantities of CO_2, and results obtained by this method show that for coarsely minced tea leaf, and for leaf that has undergone mechanical rolling in the factory, anaerobic fermentation is very largely inhibited. Complete inhibition is not observed but the damage during a coarse mincing or in the machines used for rolling tea leaf in the factory is not so complete as in a very fine mince.

Table 13. *Effect of mechanical injury on rate of anaerobic fermentation*

	ml. CO_2/100 g. fresh leaf/hr. in N_2
Fresh green leaf	8·60 (7·02–9·46)
Fresh leaf coarsely minced in a meat chopper	3·26, 4·06
Withered leaf hand-rolled for 1 hr.	0·24
Withered leaf factory-rolled for 30 min.	0·50 (0·33–0·80)
Withered leaf factory-rolled for 70 min.	0·29 (0·16–0·40)

The above figures for fresh and for factory-rolled leaf are each average values for 5 separate determinations. Destruction of the power to undergo anaerobic fermentation by rolling the withered leaf, whether mechanically or by hand, is high (90–98 %). A coarse mincing in a meat chopper is not so effective and many intact pieces of tissue can be picked out from the chopped up leaf. Broadly speaking the greater the extent of mechanical damage the lower the rate of anaerobic fermentation.

Discussion

The nature of the oxidase in tea leaf. As tea fermentation is considered to result from the disorganization of normal respiration, it is a matter of some importance to establish that the respiration of the tea leaf differs in no striking way from the respiration of other leaves. The analogy with barley, as far as the effects of carbohydrate impoverishment on respiration are concerned, is particularly striking as the two species are by no means closely related.

The effects of poisons on tea leaf respiration are also very similar to those found for other leaves. Thus James & Hora [1940] have shown that $M/500$ HCN causes 64 % inhibition of the respiration of barley leaves, and that $M/50$ HCN is required for complete inhibition. Further resemblances to tea were shown by the lag period before the HCN exerted its full effect, and the rise in the R.Q. to a value greater than unity as a result of HCN poisoning.

According to Ross [1938] the respiration of *Nitella* is only 37 % inhibited by $M/1000$ HCN. For carrot leaf [Marsh & Goddard, 1939, 1] inhibition by $M/1000$ HCN of the respiration amounts only to 61–72 % and even with $M/100$ HCN inhibition is by no means complete.

For such leaf tissues as have been investigated, in which the cytochrome system is probably operating, inhibition by cyanide is not always complete and often does not reach its maximum effect until the concentration of poison is $M/100$. This is in marked contradistinction to yeast where $M/2000$ HCN brings about complete inhibition.

For tea this relatively low sensitivity to cyanide persists in simple enzyme-substrate systems, and it appears that the oxidase system is less sensitive to cyanide than the cytochrome system from yeast. Alternatively there may be more than one oxidase system in the tissue, one of which is cyanide-stable.

This latter alternative deserves careful consideration. Boswell & Whiting [1938] have already concluded that the catechol oxidase system accounts for only two-thirds of the respiratory activity in potato tubers. The presence of a cyanide-stable oxidase system in carrot leaf, which increases with maturity, has also been deduced by Marsh & Goddard [1939, 1] who found that in the fully mature leaf, cyanide may sometimes stimulate respiration. For tea the maturer leaves are less sensitive to cyanide inhibition, so we have here some slight evidence in favour of a cyanide-stable oxidase, the relative activity of which increases with the age of the tissue.

There is another possible explanation of the low sensitivity of respiring plant tissues to the inhibitory effect of cyanides which, we believe, has not as yet been put forward. Jones [1920] stated that plant tissues generally give a more intense reaction for Fe than do animal tissues, and Ingalls & Shive [1931] demonstrated that only a small fraction of the Fe content is water-soluble. Miller [1938] states that the greater part of the Fe in plants is immobile, so it appears that a considerable portion of the Fe may be in complex organic form. The amount of this Fe is considerably in excess of that which can be accounted for by the cytochrome system. We suggest that this "fixed" Fe is able to form complexes with cyanide, so that with competition for cyanides between this fixed Fe and the respiratory system, considerably more cyanide is required for complete suppression of respiratory activity. No definite evidence can as yet be advanced in favour of this hypothesis but it is interesting to note that the less cyanide-sensitive 5th leaves of the growing tea shoot have a significantly higher Fe content. Mr P. B. Sen Gupta of this laboratory has determined the Fe contents of the 1st and 5th leaves of the growing shoot and found them to be 0·088 and 0·112% of dry matter respectively.

The CO inhibition of tea oxidase and its reversal by light is a characteristic of the cytochrome system. Similar behaviour has also been recorded for carrot respiration (leaf and root) by Marsh & Goddard [1939, 1] and for several plant tissues including the leaves of tobacco, plum and oleander [Kempner, 1936]. A relatively low inhibition by cyanide of leaf respiration may therefore be associated with a light-reversible inhibition by CO of the same order as that found for yeast respiration.

Further evidence for the presence of a cytochrome-like system in tea leaf is provided by the catholicity of substrates oxidized by a tea oxidase preparation. The range of substances oxidized is too varied to be in accordance with current views on the specificity of an enzyme towards its substrates. On the other hand, this behaviour is what would be expected were the cytochrome system operative, as the method of preparation of the tea oxidase is such that cytochrome would be found in the final product. By the same token catechol oxidase is unlikely to be present, as the method of alcohol precipitation used in the preparation of the crude enzyme would yield a product free, or nearly free, from such alcohol-soluble carriers as catechol or its derivatives.

It may therefore be concluded that either the cytochrome system or one very closely resembling it is responsible both for respiration of the tea leaf and for tea fermentation.

The effect of mechanical injury on anaerobic fermentation. Anaerobic fermentation is quite easily demonstrable in the tea leaf either by depressing the rate of aerobic respiration by inhibitors or by measuring the CO_2 output in an atmosphere of N_2. The behaviour of tea leaf under such conditions is very similar to that of carrot tissue [Marsh & Goddard, 1939, 2] and barley [James & Hora, 1940].

James & Arney [1939] have recently demonstrated that phosphate esters play essentially similar parts in the respiration of barley to those established in the case of yeast; hence it does not seem unreasonable to conclude that the role of cozymase is the same in anaerobic fermentation in both yeast and plant tissues.

Now the experimental results recorded in this communication show without doubt that extensive mechanical damage to the tissue may almost completely destroy the ability of the tissue to undergo anaerobic fermentation under otherwise suitable conditions. This cannot be attributed to extensive inactivation of the dehydrogenase, for under aerobic conditions, where oxidation products of

tea tannin may function as H-acceptors [Roberts & Sarma, 1940], carbohydrate oxidation is quite evident, and such oxidation requires the co-operation of several dehydrogenases. The failure of the mechanically damaged tissue to undergo anaerobic fermentation must therefore be attributed to an inhibition of one of the H-acceptors.

On *a priori* grounds it would seem that the H-acceptor inactivated by extensive damage to the tissue must be cozymase (or possibly coenzyme II, or both) as it is difficult to visualize the inactivation of any other H-acceptor which would have such different effects on the rate of carbohydrate breakdown under aerobic and anaerobic conditions. It is hoped when supplies of cozymase become available, to subject these conclusions to experimental verification.

Summary

The respiration of tea leaf has been studied. The R.Q. for fresh leaf is close to 1·0 and decreases as a result of carbohydrate impoverishment. Rate of respiration, but not oxidase activity, is significantly depressed as a result of starvation in the absence of light.

Respiration and tea fermentation require $M/100$ HCN for maximum inhibition. Tea oxidase-substrate systems have the same cyanide sensitivity. It is suggested that the low cyanide sensitivity of plant oxidases in general may be due to the presence of other organic Fe compounds in the tissue which compete with the oxidase system for the cyanide.

The CO inhibition of the tea oxidase is reversed by light. This, and the wide range of substrates oxidized, suggests that the tea oxidase is a cytochrome oxidase.

Anaerobic fermentation is demonstrated in tea leaf after cyanide inhibition or in an atmosphere of N_2. Extensive damage to the tissue almost completely destroys its ability to undergo anaerobic fermentation. As dehydrogenase activity is not affected to the same extent it is concluded that the effect of injury to the tissues is to inactivate the coenzymes concerned in carbohydrate oxidation.

The authors wish to thank Mr P. H. Carpenter for his interest in this work, and the Indian Tea Association for permission to publish these results.

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179. TANNINS AS HYDROGEN CARRIERS IN BIOLOGICAL OXIDATION

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(*Received 10 October 1940*)

Evidence has been presented [Roberts, 1939; 1940] which indicates that the effect of extensive bruising on tea leaf is to decrease the activity of the H-carrying coenzymes (cozymase). In the so-called tea fermentation which follows this bruising, it was established that carbohydrate oxidation, as measured by the rate of CO_2 production, parallels tannin oxidation. Further, if excess ascorbic acid be added to the system the primary oxidation product of tea tannin is reduced again immediately on its formation, and until the whole of the ascorbic acid has been oxidized no carbohydrate oxidation is apparent. These observations led to the conclusion that in tea fermentation, the tannin acted as one of the H-carriers in the oxidative stages of carbohydrate breakdown.

The possibility that cozymase was completely or almost completely inactivated by mechanical damage to the leaf was not considered, as it was believed that an *o*-quinone could not function as a H-acceptor in the absence of cozymase (Co). The later stages in the reactions in dilute suspensions of minced tea leaf were therefore considered to be

$$\text{Tannin } o\text{-quinone} + CoH_2 \xrightarrow{\text{dehydrogenase}} \text{tannin} + Co$$

$$Co + \text{substrates} \longrightarrow CoH_2 + \text{reaction products.}$$

Owing to the decrease in activity of cozymase as a result of the mechanical damage it was believed that the *o*-quinone of tea tannin was not so readily reduced and that there was in consequence a much greater tendency for it to be removed from the sphere of the reaction by undergoing irreversible condensations.

Since the publication of this work, fresh evidence has been brought forward by Dixon & Zerfas [1940] which conclusively shows that *o*-quinone requires the presence of cozymase for it to function as H-acceptor for the alcohol- and malic-dehydrogenases of yeast.

$$o\text{-quinone} + CoH_2 \xrightarrow{\text{dehydrogenase}} \text{catechol} + Co$$

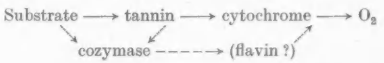
$$\text{alcohol} + Co \longrightarrow \text{aldehyde} + CoH_2.$$

However, as a result of work from this laboratory [Deb & Roberts, 1940] on the measurement of anaerobic fermentation in mechanically damaged tea leaf, it has been shown that in all probability there is an even greater destruction of cozymase activity in bruised tea leaf than was formerly visualized. It would appear that the residual cozymase activity in finely minced tea leaf is too low to account for more than a part of the carbohydrate oxidation that actually takes place, and consequently the possibility must be envisaged that the *o*-quinone of tea tannin can function directly as H-acceptor without the aid of cozymase.

A difference of this type between *o*-quinone itself and the *o*-quinone of tea

tannin is perhaps not surprising, as the chroman ring undoubtedly has a great effect on the reactivity of the catechol group in the tea tannin molecule, witness the much greater rate of oxidation by tea oxidase of tea tannin in comparison with catechol and pyrogallol [Harrison & Roberts, 1939].

In tea fermentation therefore the most likely path of H-transfer is in accordance with the following scheme:



The dotted arrows represent alternative paths for H transfer, but these will be of secondary importance in comparison with the main path, which utilizes only tannin and cytochrome as carriers between substrate and O_2. Not all of the tannin *o*-quinone will be effective as a H-acceptor, as this substance has a strong tendency to undergo irreversible condensation to form the deeply coloured tannin products, characteristic of fermented tea. Eventually the greater part of the tannin will have been converted into such complex condensation products, and oxidation of carbohydrate will come practically to a standstill. The R.Q. of the reaction will be a measure of the proportion of the tannin oxidation product functioning as a H-acceptor.

The *o*-quinone of a tannin is in all probability produced by direct oxidation by the cytochrome system. The actual oxidation of the tannin is believed to be non-enzymic and hence the tannin concentration may determine the rate of oxidation to a very large extent.

Where tannin is the principal substance oxidized by the cytochrome system, i.e. when the normal system of H-acceptors has been inactivated by the disorganization that follows extensive damage to plant tissue, we may expect a correlation between tannin content and the rate of O_2 uptake by the damaged tissue, so long as the tannin concentration remains a limiting factor. If the tannin content is such that the rate of reduction of oxidized cytochrome is equal to the maximum rate of oxidation of cytochrome by its oxidase, any increase in tannin concentration will have no further accelerating effect on the rate of O_2 uptake. The fact, previously established by Harrison & Roberts [1939], that addition of tea tannin to a suspension of 200 mg. finely minced tea leaf in 3 ml. water produces no acceleration in O_2 uptake, shows that in this particular case the limiting concentration has been reached. On the other hand the relatively short period for which minced tea-leaf suspensions maintain their original rate of O_2 uptake ($-Q_{O_2}$ for 0–10 min. is almost invariably greater than $-Q_{O_2}$ for 10–20 min.) suggests that this limiting concentration is not greatly exceeded when 200 mg. minced tea leaf are suspended in 3 ml. water.

The tannin contents of different samples of tea leaf do not differ sufficiently to establish a correlation between tannin content and rate of O_2 uptake in a fine mince. The less satisfactory method of varying the tannin content by using leaf from different plants was therefore employed. This has the compensation of extending the results obtained for tea to other plant tissues, many of which show similar effects to tea on mincing or otherwise damaging the tissue.

Experimental

Leaf tissues. Tannin contents of leaf samples were determined by the Lowenthal process in a 1 hr. infusion. Each tannin probably has a characteristic factor of its own but to avoid determining a variety of such factors, which in

itself would be a major research, it was assumed that 1 ml. of N $KMnO_4$ would be equal to 0·0416 g. of tannin (the tea tannin factor). The figures recorded for tannin percentages are therefore purely arbitrary, but probably represent the relative amounts of tannin in the various tissues with fair accuracy.

Rates of O_2 uptake in a very fine mince were determined manometrically. Minced leaf equivalent to 45–50 mg. dry wt. was suspended in 3 ml. water. The temperature of the bath was 36°, this being the lowest practical in Assam during the hot months. Where CO_2 was determined, the direct method of Warburg was used: in four vessels O_2 uptakes were measured with a CO_2 absorbent (KOH on filter paper) and in four other vessels containing comparable samples of tissue the CO_2 absorbent was omitted. This method, although shown to be inaccurate for mammalian tissue slices, gives quite good results for plant tissues. Retention of CO_2 by tissues seems to be low, and the buffer retention is negligible, as the tissues are suspended in distilled water. Turner [1938] has shown this method to give values for the R.Q. of carrot slices almost identical with those obtained by the more complicated method of Dickens & Šimer.

The rate of O_2 uptake ($-Q_{O_2}$) is defined as the initial rate in μl. O_2/hr. for 1 mg. dry wt. of tissue. The figures quoted in Tables 2–6 represent uptakes in μl. O_2/100 mg. tissue (fresh weight).

The results obtained for young leaves from many different species are summarized in Table 1.

Table 1

Family	Species	$-Q_{O_2}$	% tannin (dry wt.)	R.Q.
MONOCOTYLEDONS:				
Cannaceae	*Canna* (cultivated variety)	11·0	5·8	—
Liliaceae	*Hemerocallis fulva*	3·0	0·0	—
Amaryllidaceae	*Eucharis grandiflora*	1·0	0·0	—
	Hippeastrum equestre	1·8	0·0	—
Gramineae	*Zea mays*	2·9	0·0	—
DICOTYLEDONS:				
Tropaeolaceae	*Tropaeolum majus*	2·2	0·0	0·9
Theaceae	*Eurya accuminata*	4·4	3·3	0·31
	Pyrenaria barringtoniaefolia	18·5	13·8	0·07
	Schima wallichii	6·1	10·7	0·29
	Gordonia excelsa	2·0	14·9	0·44
	Camellia thea	20·0	22·0	0·36
	C. drupifera	4·5	27·7	0·37
Myrtaceae	*Eugenia balsamea*	18·2	18·9	0·56
	E. jambolanea	13·0	16·6	0·32
Malvaceae	*Hibiscus rosasinensis*	1·6	0·0	1·0
Umbelliferae	*Daucus carota*	13·8	1·7	—
Apocynaceae	*Allamanda cathartica*	5·1	1·6	0·36
Rubiaceae	*Gardenia florida*	2·3	2·2	1·1
Compositae	*Coreopsis* (annual spp.)	14·8	3·8	—
	Dahlia	18·0	1·2	—
	Eclipta alba	22·5	6·8	0·54
Bignoniaceae	*Bignonia venusta*	4·8	0·9	0·30
	B. aequinoxalis	5·0	1·0	0·39

An examination of this table shows that, broadly speaking, leaves with a low tannin content show a very slow rate of O_2 uptake in a fine mince, whereas leaves of high tannin content show a comparatively rapid rate. In all six cases where tannin is not present in the tissues, the rate of O_2 uptake in the finely minced tissue is very low. The correlation coefficient unfortunately falls short of significance, $r = 0{\cdot}356$ (0·396 required for significance when $P = 0{\cdot}05$).

This is perhaps not surprising, as the correlation may be obscured by several

factors. Thus the effect of fine mincing on the rate of O_2 uptake varies markedly with the type of tissue. In the case of young shoots from tea (*Camellia thea* Link), the finely minced petiole shows a much higher rate of O_2 uptake than the blade, despite the former's lower tannin content. Some of the inconsistencies in the above table may be explained in terms of a greater proportion of petiole to blade tissue in some of the more rapidly respiring cases. In addition to lack of uniformity in the tissue resulting in different reactions of respiratory activity to damage, another factor, the toxic effect of the tannins, must be considered.

In the two cases *Gordonia excelsa* and *Camellia drupifera*, where tissues of high tannin content show a low $-Q_{O_2}$ in a mince, the strength of the tannins is of a high order. Unlike tea tannin they can precipitate gelatin directly, without the assistance of acid and salt, and solutions of these tannins are sufficiently toxic to depress the rate of O_2 uptake in other minced tissues, particularly tea.

Such strong tannins may fairly be considered to be toxic even to their own oxidase systems, and we consider it legitimate to exclude these two results when calculating the correlation coefficient between $-Q_{O_2}$ and tannin content. The correlation coefficient now becomes 0·6160 which is easily significant for $P=0{\cdot}01$.

The majority of the tanniferous leaves show O_2 uptake and CO_2 output curves similar to those of tea [cf. Roberts, 1939]. The initial uptake is rapid, slowing down as tannin oxidation becomes complete. The R.Q. usually varies from 0·3 to 0·5 indicating that condensation of the *o*-quinone molecule is rather more likely than its functioning as a H-acceptor. When the greater part of the tannin molecules have been transformed into oxidized and condensed tannin products, both O_2 uptakes and CO_2 outputs sink to low levels.

The non-tanniferous leaves not only show very low initial rates of uptake, but this rate is not maintained for long. It would seem that the reaction comes to a standstill after all the carriers are in their oxidized form. The R.Q. in such cases is found to be close to unity. Typical results for two representative species are summarized in Table 2.

Table 2

		Min.						
		15	30	45	60	120		
Tropaeolum majus	O_2 (μl.)	8	13	14	16	19		
	CO_2 (μl.)	7	10	11	12	15		
		Min.						
		15	30	45	60	75	90	105
Eugenia jambolanea	O_2 (μl.)	49	95	133	171	192	217	237
	CO_2 (μl.)	8	18	34	48	57	67	76

Not all species fall into these two clearly differentiated types. Thus *Gardenia florida* (Table 3) shows a continuous uptake of O_2 and evolution of CO_2 for the finely minced leaf tissue.

Table 3

		Min.					
		15	30	45	60	90	120
Gardenia florida	O_2 (μl.)	15	25	36	45	56	72
	CO_2 (μl.)	14	25	36	46	63	80

This is obviously a case where mincing has not seriously inactivated the reducing powers of the tissue. The tannin in *Gardenia* may not condense readily on oxidation, coenzyme inactivation may be less apparent, or alternatively

dehydrogenase activity may be high enough to reduce any *o*-quinone immediately on formation. It is impossible to decide between these three alternatives at the moment.

At the other extreme, we have *Pyrenaria barringtoniaefolia* where the R.Q. is 0·07. Here very little carbohydrate oxidation is apparent and most of the *o*-quinone must condense before it has had a chance of functioning as H-acceptor.

Minced leaf from the carrot (*Daucus carota*) and from the three Compositae investigated, show much higher initial rates of O_2 uptake than would be expected from their tannin contents. This high rate is only transient and soon falls to a low value. With *Eclipta alba* the rate of uptake is initially so high that oxidation may be nearly complete before the minced tissue is weighed out into the Warburg vessels. Samples of this plant contain a large proportion of succulent stalk, and as was found for tea, such tissue undergoes very rapid oxidation. *Eclipta alba* is also of interest as the tannin condensation product is dark blue in colour unlike the more usual brown of these substances. This blue colour is made use of locally as an ink, and also for tattooing and hair dyeing.

Fruit tissues. The effect of fine mincing on fruit tissues is similar to that on leaves. Thus the R.Q. of apple as measured for thin slices, is reduced to 0·60 on fine mincing, and the mince rapidly darkens in colour. The fruit of *Phyllanthus emblica* behaves similarly. Here the R.Q. is 0·44 after mincing, and the rate of O_2 uptake in the mince is 82 μl./hr./100 mg. tissue, as against 14 μl. for the apple. This higher rate is paralleled by the relatively high tannin content (10 % of dry wt.) of the latter fruit. The tannin condensation products of this fruit, like those of *Eclipta alba*, are blue.

Specificity of tannins as carriers. It might be expected that addition of a tannin to finely minced non-tanniferous leaves such as *Tropaeolum*, *Hibiscus* etc. would stimulate the rate of O_2 uptake. Neither of the above species reacts significantly in this way as shown by the results in Table IV for *Tropaeolum*.

Table 4

	Min.				
	15	30	45	60	90
	O_2 uptake (μl.)				
Tropaeolum	10	11	12	13	14
Tropaeolum + tea leaf infusion	13	14	15	16	17

The failure of added tea tannin to stimulate O_2 uptake was further investigated and it was found that the oxidase system of a given plant, while generally capable of promoting oxidation of tannins from its own species, has comparatively little action on foreign tannins.

This is shown quite clearly in the following experiment. Finely minced portions of leaf, from tea and from *Eugenia balsamea*, were suspended in 2 ml. of water in Warburg vessels and shaken for 50 min., at the end of which time O_2 uptake had slowed down considerably. Fresh substrate in the form of green leaf infusions from either of the two species was then added and the uptakes recorded over a further period. Tea tannin stimulated the O_2 uptake of tea leaf to a much greater extent than that of *E. balsamea*, while the *E. balsamea* tannin had a greater effect on the O_2 uptake catalysed by its own enzymes (Table 5).

For tea the oxidation of tea tannin is not directly an enzymic process but is brought about by oxidized cytochrome [Roberts, 1940; Deb & Roberts, 1940]. Enzymic specificity therefore is unlikely to account for these differences, and it is suggested that plant tissues possess some sort of "immunity" to the toxic effect

Table 5. *O_2 uptake after addition of fresh substrate*

		Min.				
Minced tissue	Infusion added	15	30	45	75	105
Tea	Tea	68	108	125	146	159 μl.
Tea	*E. balsamea*	25	42	50	63	73 μl.
E. balsamea	Tea	23	44	61	86	102 μl.
E. balsamea	*E. balsamea*	86	115	134	147	158 μl.

of their own specific tannins, and that foreign tannins in consequence have a greater inhibitory effect on oxidase activity. The general toxic effect of tannins on enzyme activity is well known.

The inactivating effect of a foreign tannin on oxidase activity may in some cases be reversible. Thus on successive additions of infusions of *E. balsamea* and tea leaf to fermented tea leaf suspensions, O_2 uptake is accelerated by the second but not by the first addition of fresh substrate. This effect is illustrated in Fig. 1.

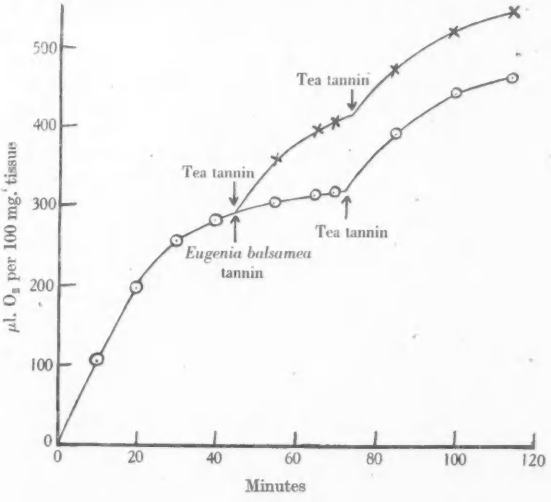


Fig. 1.

E. balsamea infusions when added to freshly minced tea leaf suspensions have no significant effect on the initial rate of O_2 uptake, although they do actually increase the total uptake. However, at the end of 90 min. comparatively little of the total *E. balsamea* tannin has been oxidized. Uptakes are much higher after 90 min. when tea infusions are added to the system in this way.

Camellia drupifera tannin, however, significantly depresses the rate of tea fermentation, the $-Q_{O_2}$ falling from 18·9 to 15·3 as a result of the addition of

Table 6

	Min.						
	10	20	30	45	60	75	90
	O_2 uptake (μl.)						
Tea fermentation	63	110	145	164	170	177	181
Tea fermentation + *E. balsamea* infusion	62	112	153	190	200	210	217

1 ml. of a *C. drupifera* infusion (20 g./500 ml.) to 200 mg. tea leaf suspended in 2 ml. water. With partly fermented tea leaf the inhibition by *C. drupifera* infusions exceeded 50 %. Unfortunately further experiments with *C. drupifera* could not be carried out as supplies of fresh leaf were no longer available.

Discussion

The low O_2 uptakes by minced suspensions of non-tanniferous leaves, and the establishment of a correlation between the tannin content of a leaf and its rate of O_2 uptake in a fine mince confirm the belief that tannin is an essential carrier in oxidation processes taking place in minced leaf tissue. Taking into account the observations quoted elsewhere, which indicate that cozymase is almost completely inactivated by extensive damage to the tea leaf, we may conclude that, as carbohydrate oxidation is apparent in fermenting tea leaf, the *o*-quinone of tea tannin may function as a H-acceptor for dehydrogenase systems without the assistance of cozymase. This apparently distinguishes the *o*-quinone of tea tannin from *o*-quinone itself.

This conclusion however is not in accordance with all the experimental facts. Previous results from this laboratory [Harrison & Roberts, 1939] have shown that addition of catechol or pyrogallol to a suspension of finely minced tea leaf increases the output of CO_2. We cannot be certain that this additional CO_2 comes from carbohydrate oxidation but until further evidence is available it would be premature to conclude that *o*-quinone added to tea leaf should behave in the same way as in the systems investigated by Dixon & Zerfas.

Although there are many analogies between the biological oxidation systems present in yeast and animal tissues on the one hand, and in leaf-tissue on the other, we are not yet justified in assuming that the same carriers are required, and the role of flavoproteins and pyridine nucleotides in plant respiration deserves intensive study.

Summary

A correlation has been found, rejecting two anomalous cases, between the tannin content of leaf tissue and its rate of O_2 uptake in a fine mince. This confirms previous ideas that tannins function as H-carriers in biological oxidation systems.

The properties of the *o*-quinone of tea tannin as H-acceptor are discussed. Apparently, it does not require the presence of cozymase.

The apparent specificity of a plant oxidase for tannins from its own species is considered to be due to an "immunity" of the enzymes of a given species to the toxic effects of its own tannins.

The authors wish to thank the Director, Mr P. H. Carpenter, for his encouragement throughout the course of this work and the Indian Tea Association for permission to publish these results.

Dr W. Wight's assistance and advice on the botanical side of this work is gratefully acknowledged, and thanks are also due to Mr Percy-Lancaster of the Royal Agri-Horticultural Society of India for identification of some of the species used.

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180. METHODS FOR THE VOLUMETRIC ESTIMATION OF TEA TANNIN IN GREEN-LEAF AND BLACK TEA. A NEW ALKALINE PERMANGANATE METHOD

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(*Received 10 October 1940*)

In connexion with work in progress in this laboratory on tea fermentation, a volumetric method for the determination of tea tannin has been devised, which is free from many of the disadvantages attending other methods in common use. This method has already enabled us to confirm some of our findings on the extent of oxidation of tea tannin in tea fermentation. It also makes it possible to estimate the amount of tannin in black tea with much greater precision than was formerly possible, and it is hoped that it will facilitate any work on made tea that may be undertaken in the home country. The method may also prove to be of use to biochemists who are interested in tannins other than those obtained from tea.

In the absence of any specific volumetric reagent for tea tannins, these substances are determined in tea infusions by an oxidimetric titration of the infusion followed by a similar determination on another portion of the solution from which tannin has been quantitatively removed. The difference between the "total" and the "non-tan" titres gives the tannin titre.

The infusion is prepared by boiling 5 g. tea or 20 g. fresh green leaf with 400 ml. water for 1 hr., cooling and making up the volume of the extract to 500 ml. Fresh green leaf contains from 75 to 80 % moisture so that the above quantities are approximately equivalent to each other.

The separation into tannins and non-tans is usually effected by gelatin. The following reagents are required.

Gelatin solution. 25 g. gelatin are soaked for 1 hr. in saturated NaCl solution. The mixture is then warmed until the gelatin has dissolved and after cooling the solution is made up to 1 l. with saturated NaCl.

Acid NaCl. 25 ml. of conc. H_2SO_4 are added to 975 ml. saturated NaCl solution.

100 ml. of a tea infusion are mixed with 50 ml. of the gelatin solution, 100 ml. of acid NaCl and 20 g. of powdered kaolin. After shaking for several min. and allowing to settle, the mixture is filtered and an aliquot of the filtrate withdrawn for titration. 25 ml. of the non-tan filtrate are equivalent to 10 ml. of the original infusion.

The Lowenthal and alkaline iodine methods

Hitherto two procedures have been in common use in Tea Research laboratories for the volumetric determination of tannins, the Tocklai modification of the Lowenthal method, and Shaw's iodine method. A Lowenthal technique has been adopted as a tentative method by the Association of Official Agricultural Chemists [1936].

The Tocklai-Lowenthal method. The following reagents are required: (1) $N/25$ $KMnO_4$; (2) 1·5 g. indigo carmine dissolved in 1 l. water containing 50 ml. H_2SO_4.

A 10 ml. aliquot of the infusion is mixed with 25 ml. of the indigo-carmine solution, and the mixture diluted to 750 ml. $KMnO_4$ is then run in from a burette 1 ml. at a time with brisk shaking. As titration proceeds the blue of the indigo-carmine passes through many shades to a final yellow with a faint pink tint at the rim. This is taken as the end-point and needs considerable judgement on the part of the operator. The titre is affected to some extent by the amount of tannin in solution, the time taken in the titration and the vigour of the shaking. Repeats often differ by 0·3 ml. or more, and the variation between different operators may amount to 1 ml.

A correction in the total titre, A, must be made for the indigo-carmine oxidized. This usually amounts to 4·0 ml. For the non-tan titre, B, a slightly higher blank of 4·5 ml. is found in practice. The tentative method of the A.O.A.C. makes no mention of this variation in the blank and arrives at the tannin titre by subtracting the non-tan from the total titre. The actual tannin titre will be $(A-4{\cdot}0)-(B-4{\cdot}5)$.

The factor for converting ml. N $KMnO_4$ into g. tannin is 0·0416 as determined by the hide powder method. This, quite fortuitously, equals the factor for gallotannic acid.

The alkaline iodine method. The method was elaborated by Shaw [1930] and has been slightly modified at Tocklai. Oxidation is effected by an excess of $N/10$ I_2 in alkaline solution, and the excess I_2 determined after a period of 30 min. The same gelatin method is used for the separation of non-tans. The end-point is of course much sharper in this method than with that of Lowenthal. The main objection to the method lies in the incompleteness of the oxidation by alkaline I_2, so that the room temperature and the time during which oxidation is taking place have a distinct effect upon the final result.

In both of the above methods oxidation of the tannin to some indeterminate product takes place. Assuming tea tannin to be a mixture of catechin and gallocatechin units [Harrison & Roberts, 1939] with a mean mol. wt. of 300, it may be calculated that in the Lowenthal method some 7·1 equiv. O_2 are consumed in the oxidation of 1 mol. tea tannin, whereas in the modified Shaw method 16·7 equiv. are used up.

For complete oxidation to CO_2, an equimolecular mixture of these two catechins would require 60 equiv. O_2 per mol. Most of the disadvantages of the two methods so far described are really due to the uncertainty of the extent of oxidation of tannin that takes place during the estimation. They may, however, be used to determine the amount of tannin in green tea leaf with fair accuracy if the conditions during the whole estimation are very carefully standardized. For manufactured (black) tea, however, they give very erroneous results, and the two methods give quite different figures for tannin contents, if the factors determined for green leaf are applied (Table 1).

Table 1. *Tannin titres for fresh and fermented tea leaf*

	Lowenthal		Alkaline I_2	
	ml. 0·04 N $KMnO_4$ per g. dry wt.	% tannin	ml. 0·04 N I_2 per g. dry wt.	% tannin
Fresh leaf	139·2	23·2	302·0	21·4
Leaf fermented 2½ hr.	88·7	14·8	247·4	17·5

It is usual to find analyses recorded for made tea, carried out by the official Lowenthal method, stating that the tannin content is from 12 to 14 %. Such a drop in the actual tannin content as a result of fermentation cannot be established. There is certainly a fall in the water-soluble tannin as a result of tea-fermentation [Harrison & Roberts, 1939] but not more than 15–20 % of the total amount of tannin in the green leaf is rendered insoluble in this way. A large decrease in the water-soluble tannin as a result of fermentation would be accompanied by a corresponding decrease in the total water-soluble solids. There is indeed a decrease here, but the average for made tea is 41 % against 45 % for green leaf, so even if the whole of the decrease in water-soluble solids were due to decreased solubility of the tannin, the fraction of the tannin thus made insoluble would not exceed 20 % of the whole.

It must be remembered that the oxidation product of green leaf tea tannin is still a tannin, and presumably this tannin, being partially oxidized, will require less $KMnO_4$ or I_2 to oxidize it. Different factors are therefore required for the tannins in made tea infusions, but even if allowance is made for the oxidation of the tannins and the proportion of these bodies which have become insoluble (by combination with leaf protein), the decrease in the Lowenthal and alkaline I_2 titres is still too great to be accounted for. Further, if tannins are incubated with tea oxidase solutions, the decrease in the tannin titre is greater than the equivalent O_2 uptake, and under such conditions none of the tannin has been rendered insoluble.

An explanation of these difficulties has been found in the condensation of the tannin bodies after oxidation. It is known that the condensation of catechins is greatly accelerated by oxidation of the molecule [Freudenberg, 1933] and from the kinetics of tea fermentation it follows that some irreversible change must occur in the primary oxidation product of tea tannin. Further, the varying liquoring characters of black tea can be very satisfactorily explained if different degrees of condensation of the oxidized tannins are assumed.

Now highly condensed products, such as the tannins in black tea, will not be so readily oxidized as their simpler parent substances in green leaf, and it will be readily seen that in both Lowenthal's and Shaw's methods, slower oxidation means less oxidation. The same quantity of tannin will have taken up less permanganate before the indigo-carmine is completely oxidized, and in the same period of incubation, less tannin will have been oxidized by the alkaline I_2. The drop in tannin content, as a result of fermentation, would therefore seem to be apparent only, and it must be concluded that the two methods in general use give very erroneous results for the tannin content of a black-tea infusion.

Reference to Table 1 shows that the apparent fall in tannin content on fermentation is not so marked when the alkaline I_2 method is used for the estimation. This is of course due to the greater number of O equivalents used up in the I_2 titration, as the actual decrease in titre (54·6 ml. 0·04 *N* I_2) is greater than that recorded by the Lowenthal method (50·5 ml. 0·04 *N* $KMnO_4$). From this result it would appear that condensed tannins are less readily oxidized by the alkaline I_2 than they are by the Lowenthal method. The example quoted in Table 1 gives the tannin changes in coarsely minced fresh leaf after $2\frac{1}{2}$ hr. fermentation. Complete oxidation of tannin to the *o*-quinone is not realized in this time. This point must be emphasized as otherwise it would appear that the decrease in the titre is no greater than would be expected from oxidation and combination of 15–20 % of the tannins with leaf protein. Comparison of the percentages of tannin in made tea according to these two methods, and the new alkaline $KMnO_4$ method, given in Table 4 shows that Shaw's method does give low results.

Stamm's alkaline permanganate method

From the above discussion of the methods of tannin titration so far used in tea chemistry, it is evident that most of their drawbacks are connected with the indefinite nature of the end-products of oxidation. A method is required in which the tannins of either green leaf or black tea are oxidized to a definite end-point.

For green-leaf tannins cerimetry might prove practicable, but such a method would be of no use in estimating the tannins in black tea, where from 75 to 95 % of the tannins are already oxidized. Preliminary volumetric estimations with ceric sulphate have indicated that tea tannin is oxidized only to the quinone stage by this reagent, which implies that only 2 equiv. O are used per mol. of tea tannin.

Complete oxidation to CO_2 is desirable, and this has been realized with Stamm's alkaline permanganate method [Böttger, 1938], the second procedure, which involves back-titration with oxalic acid in an acid medium, being employed. For the theory of the method reference should be made to Böttger's monograph.

The success of the method was foreshadowed by the complete oxidation to CO_2 by alkaline $KMnO_4$ of catechol, pyrogallol, gallic acid and phloroglucinol all of which enter into the composition of one or other of the tea-tannins.

Reagents.

(1) 0·1 *M* $KMnO_4$ (15·803 g. per l.).

(2) 30 g. pure NaOH in 100 ml. H_2O.

(3) 0·02 *M* $KMnO_4$.

(4) Oxalic acid, containing about 34 g. $H_2C_2O_4, 2H_2O$ per litre.

(5) Strong H_2SO_4 containing 1 part of acid to 1 part of H_2O by weight.

(6) $MnSO_4, 4H_2O$, 50 g. per l.

Procedure. Infusions of 20 g. fresh green leaf or 5 g. made tea in 500 ml. are prepared. Solutions for non-tan estimation are prepared in the same way as for the Lowenthal method. A 20 ml. portion of the original infusion and a 25 ml. portion of the non-tan filtrate are each diluted to 100 ml., and 10 ml. and 20 ml. portions respectively of these diluted solutions are withdrawn for "total" and "non-tan" estimations.

The sample to be oxidized is treated with 20 ml. 0·1 *M* $KMnO_4$ and 10 ml. NaOH (2), and the mixture allowed to stand for 10 min. at room temperature (*c.* 30°) after which it is diluted to 250 ml.; 10 ml. H_2SO_4 (5), 20 ml. oxalic acid (4) and about 10 ml. $MnSO_4$ (6) are then added. The colour of the solution is quickly discharged on shaking, and after heating the solution to 50° the estimation is completed by titrating the residual oxalic acid with 0·02 *M* $KMnO_4$. The above procedure gives values, *A* and *B*, for total and non-tan titres respectively.

Reagent blanks must also be determined. The blank consumption, according to Stamm, is obtained by treating 20 ml. oxalic acid with 5 ml. H_2SO_4 and warming to 50° after which 10 ml. $MnSO_4$ and 20 ml. 0·1 *M* $KMnO_4$ are added; after decoloration the excess oxalic acid is titrated with 0·02 *M* $KMnO_4$, this final titre giving the required blank, *C*.

As in the Lowenthal method, the "non-tan" reagents blank is higher than the reagents blank just described. A blank non-tan filtrate is prepared using 100 ml. H_2O in place of 100 ml. infusion, and an equivalent amount of the filtrate to that used for the non-tan determination is withdrawn for titration. The non-tan blank *D* is generally some 0·6 ml. higher than the reagents blank *C*.

The final tannin titre is given by $(A–C)–(B–D)$.

Some of the stages in the above procedure require explanation. The taking of a large sample for the infusion, and the use of such a small aliquot is necessary for two reasons. Smaller samples than those used are apt to be unrepresentative, unless the green leaf is very finely minced, or the tea finely powdered. Further, the use of a much less concentrated infusion often results in incomplete precipitation of tannin by gelatin.

Considerable difficulty was experienced at first with the non-tan determinations which showed wide variations on replication. This difficulty was due to the oxidation by $KMnO_4$ of the HCl liberated on acidification after the alkaline $KMnO_4$ oxidation. If the solution is diluted to 250 ml. immediately after the incubation with alkaline $KMnO_4$ oxidation of HCl becomes a small and fairly constant factor, and the addition of oxalic acid may be postponed for 5 min. without affecting the titration values to a measurable extent. Although this factor is only of importance when titrating non-tan filtrates it is considered advisable to carry out the titration in the same way for both total and non-tan estimations.

The non-tan fraction consists mainly of glucose, caffeine and amino-acids. The first of these is known to be completely oxidized by alkaline $KMnO_4$ to CO_2. Oxidation of the other two substances has been shown to be incomplete, as higher titration figures are obtained by extending the time of incubation or increasing the temperature to 40°. This incomplete oxidation of some of the non-tan substances however, does not materially affect the result. The total contribution of caffeine and amino-acids to the non-tan titre does not exceed 1 ml. in the aliquot used. Independent measurements have shown that extreme variations in the time and temperature of incubation do not affect this titre by more than 0·2 ml. As total and non-tan titrations are carried out under almost identical conditions, the error due to this incomplete oxidation is unlikely to exceed a fraction of 0·1 ml., and may safely be neglected.

The greatest care must be exercised throughout the determination in burette and pipette measurements of $KMnO_4$, since every error is multiplied by 5 because of the change from 0·1 *M* to 0·02 *M* solution in the determination of the excess oxalic acid.

Calculation of results. The conversion of the tannin titre of a solution into a percentage by weight of water-soluble tannin in the sample requires a knowledge of the structure of tea tannins which we do not possess at present. Until such knowledge is available an empirical factor only can be employed.

There is good reason for believing that the tannins in tea consist largely of the simpler condensation products of epicatechin and gallocatechin. The possibility that the galloyl ester of catechin is also present in relatively large amounts is indicated by the recent work of Deijs [1939]. The number of equivalents of O necessary for complete oxidation to CO_2 of these substances is shown below:

	Mol. wt.	Equiv. O for complete oxidation	g. tannin per equiv. O
Catechin	290	62	4·68
Gallocatechin	306	60	5·10
Galloyl-catechin	442	86	5·14

It may be assumed therefore that each equivalent of O oxidizes 5 g. of tannin, without any very great error. On this assumption the tannin titre obtained for a 10 ml. aliquot of the fivefold diluted green leaf infusion must be multiplied by 0·625 to give the percentage water-soluble tannin in the original green leaf. Results obtained in this way have agreed quite closely with those obtained by the Lowenthal method.

For black tea infusions a different factor must be employed. The tannin molecule takes up 1 atom only (2 equiv.) of O as a result of enzymic oxidation. During fermentation such oxidation is about 85% complete on the average, so that the uptake of O_2 amounts to 1·7 equiv./mol. of tannin. 300 g. of tannin therefore now require only 58·3 equiv. of O for complete oxidation, neglecting changes in weight of tannins due to oxidation and condensation. As 5 g. of black tea are taken for the infusion as against 20 g. fresh leaf the factor now becomes 2·57. The tannin titre multiplied by this factor gives the percentage of water-soluble tannin in black tea. It is not usual to express this percentage in terms of dry weight. For green tea, where the tannins have not been oxidized during manufacture, the factor would be 2·50.

Results. Representative results with green leaf and made tea are quoted below.

Green leaf		ml. 0·1 *M* $KMnO_4$
Total titre	=	18·5–6·7
Non-tan titre	=	12·0–7·5
Tannin titre	=	(18·5–6·7) – (12·0–7·5) = 7·3
% tannin (wet wt.)	=	7·3 × 0·625 = 4·56%
Moisture content of green leaf	=	79·0%
% tannin (dry wt.)	=	21·7%

Made tea		ml. 0·1 *M* $KMnO_4$
Total titre	=	18·3–7·5
Non-tan titre	=	11·7–8·1
Tannin titre	=	(18·3–7·5) – (11·7–8·1) = 7·2 ml. $KMnO_4$
% tannin	=	7·2 × 2·57 = 18·50%

The average water-soluble tannin content in made tea is distinctly lower than that of fresh leaf although the figures are much higher than those found for black tea by the Lowenthal method. A drop in water-soluble tannin is also observed after tea fermentation.

Thus Table 2 records percentages of tannin as found by Stamm's and Lowenthal's methods for coarsely minced samples of fresh and fermented tea leaf. Four 20 g. samples of the coarsely minced leaf were weighed out. Two were infused at once and the other two after allowing 2½ hr. for the fermentation process to take place. The figures in the above table are the means of duplicate samples; percentages are on the original wet weight basis.

Table 2. *Tannin percentages of fresh and fermented leaf*

	Alkaline $KMnO_4$	Lowenthal
Green leaf	5·08	5·41
Fermented leaf	4·22	3·23

It has already been shown that according to the Lowenthal method the fall in the $KMnO_4$ tannin titre is considerably greater than corresponds with the uptake of O_2, even when the oxidation of the tannin takes place in aqueous solution [Roberts & Sarma, 1938; Harrison & Roberts, 1939]. The following experiment shows the relative fall in tannin titre by the Lowenthal and Stamm methods when enzyme and substrate are incubated together.

The enzyme was a solution in *M*/15 phosphate buffer *p*H 5·8 of an arbitrary amount of the alcohol precipitate of withered tea-leaf juice [Roberts, 1940], and the substrate was tea tannin prepared according to the standard Tocklai method [Harrison & Roberts, 1939]. Mixtures of 1 ml. enzyme and 2 ml. substrate (500 mg. tannin in 50 ml. H_2O) were shaken in each of 10 Warburg vessels until

the fall in the rate of O_2 uptake indicated that oxidation of the tannin was nearly complete. The united reaction products from the Warburg vessels, after suitable dilution, were then analysed for their tannin contents by the Lowenthal and Stamm methods. 20 and 5 ml. aliquots were taken respectively for these two determinations and the corresponding amounts for non-tan estimations. The initial tannin content of the enzyme-substrate mixture was determined on another solution containing 20 ml. of the tannin solution and 10 ml. enzyme, diluted to 200 ml.

Table 3

	Stamm ml. 0·1 *M* $KMnO_4$	Lowenthal ml. 0·04 *N* $KMnO_4$
Initial tannin titre	7·9	9·4
Final tannin titre	7·7	6·4

The calculated difference in tannin titre for alkaline $KMnO_4$ is 0·26 ml., so the observed difference agrees very well with that predicted.

Discussion

The foregoing results show that Stamm's alkaline $KMnO_4$ method may be adapted to the determination of tannins both in green leaf and in made tea.

The method, although tedious in comparison with that of Lowenthal, is capable of greater precision; the end-point is sharp and does not vary with the personal factor and the conditions under which the determination is carried out. For rapid methods of estimation of unoxidized tannin where great accuracy is not called for, the Lowenthal is more convenient, but for the estimation of tannin in fermented tea the Stamm procedure is the only method which gives results approaching accuracy. The Lowenthal titre varies so much with the conditions of fermentation that it can give no reliable measure of the tannin content. Cases, particularly those where slowly fermenting varieties of tea are concerned, are frequently met where one tea, which possesses the highest Lowenthal titre of several under investigation, actually has the lowest true tannin content.

In conclusion it may be of interest to record percentages of tannin for two samples of black tea, as determined by the three methods here reviewed. The first tea was unusually high in tannin, and only some 70 % of its tannin had been oxidized during fermentation. A longer period of fermentation would have accentuated the differences found by these methods. The second tea had been fermented for $5\frac{3}{4}$ hr. at 90°, and must be considered distinctly overfermented.

Table 4

	Tannin %		
Sample	Lowenthal	Shaw	Stamm
A	16·0	18·9	20·2
B	13·8	15·0	18·0

The above method applies not only to tea tannin but has been successfully used in the estimation of tannins in other plant tissues.

Summary

Current methods for the volumetric estimation of tannins are reviewed. Errors in such determinations are mainly due to the arbitrary nature of the end-point which corresponds with no definite end-product of oxidation.

For unoxidized tannins these methods give fairly accurate results but they are completely unreliable when oxidized tannins are being estimated, as the titration is carried to a different end-point owing to decreases in the rate of oxidation of the tannin bodies as a result of condensations following oxidation.

The Stamm alkaline $KMnO_4$ method gives a much more accurate result for oxidized tannins, as in this method the tannins are oxidized completely to CO_2.

The authors wish to thank the Director, Mr P. H. Carpenter, for his interest in this work, and the Indian Tea Association for permission to publish these results.

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181. THE IMPORTANCE OF AMINO-ACIDS AS YEAST NUTRIENTS

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(*Received 10 October 1940*)

At the time of the earliest chemical studies of yeast propagation it was supposed that protein material could serve effectively for yeast nutrition. The formation of higher alcohols was later traced to the presence of specific amino-acids in the culture medium.

The discovery of "bios" in Ide's laboratory threw doubt on the efficacy of material strictly of protein origin, but the existence of "bios" was doubted and Pringsheim [1906], thinking that material of protein origin constituted the alleged mysterious substance, published an extensive article on the nitrogen nutrition of yeast [Pringsheim, 1907] without so much as referring to "bios" investigations. His attitude no doubt had much to do with the abandonment of "bios" studies from 1906 to 1919, when one of us postulated a relationship between "bios" and vitamin B [Williams, 1919].

Recognizing the existence of specific vitamin-like nutritional factors, quite distinct from amino-acids, Williams and co-workers have pointed out the importance of asparagine [Williams, 1920] and aspartic acid [Williams & Rohrman, 1936] as yeast nutrients and Miller and co-workers have found leucine [Miller, 1936] to be a nutrient factor of importance for certain yeasts. Still more recently, Nielsen & Hartelius [1937] have studied the effect of adding amino-acids to a medium containing a small amount of beerwort, and have shown that for the yeast under investigation a number of amino-acids have individually toxic effects under these conditions but collectively have a beneficial action, increasing the yeast crops by as much as 84 % under the experimental conditions used.

In all of these studies the investigation of the effects of amino-acids could not be made adequately because sources of the nutrient factors, free from amino-acids, were not obtainable. In the latter study one strain of yeast only was investigated.

Since the isolation of pantothenic acid, biotin, aneurin and vitamin B_6 (as well as inositol), all of which are yeast nutrient factors, these substances have all been available in our laboratory [Williams *et al.* 1940] and it becomes possible to determine more definitely how important amino-acids are for the nutrition of yeast and especially to investigate the differences which exist between different strains of *Saccharomyces cerevisiae*. Our experience led us to expect striking differences and to the opinion that a study of a single strain of yeast could not be taken as generally significant.

Materials

The twenty amino-acids used were Eastman products: asparagine, alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, hydroxyproline, *iso*leucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine,

tryptophan, tyrosine and valine. Solutions of each amino-acid containing 0·5 mg. per ml. were prepared and used in the individual tests. Solutions were also prepared containing equal amounts of the above twenty amino-acids, one with 5 mg. total per ml. (250 μg. of each) and one with 0·5 mg. total per ml. (25 μg. of each).

The casein hydrolysate was prepared as follows: 10 g. of "B"-free casein (Eimer and Amend) was autoclaved 6 hr. with 100 ml. of 6N H_2SO_4. After removal of the sulphate with barium hydroxide, 200 mg. tryptophan were added to replace that destroyed in hydrolysis. Dilutions were then made to yield solutions equivalent to 5 and 0·5 mg. of the original casein per ml.

The basal medium was that of Williams & Saunders [1934], containing inositol but excluding asparagine (and aspartic acid). In addition a supplement of nutrient factors was added. These were as follows with the amounts of each added per culture: aneurin 1 μg., vitamin B_6 2 μg., biotin[1] 0·0005 μg., calcium pantothenate 0·5 μg., riboflavin 2μg., uracil 2 μg., choline 3 μg., acetylcholine 2 μg., ethanolamine 10 μg., nicotinamide 10 μg. The effects of the first four of these on certain strains of yeast have been studied by Williams *et al.* [1940]. The last six compounds have been found to be somewhat stimulating for certain yeasts in unpublished work in this laboratory.

Yeast seedings were in most cases obtained from cultures maintained in this laboratory for several years. Five are strains of *Saccharomyces cerevisiae*:

F.B.—Fleischmann Bakers'.
2190—British Type Culture Collection (Narayanan).
H. 1—Wildiers' yeast (Hansen 1).
G.M.—Gebrüder Mayer.
O.P.—"Old Process" Fleischmann.

A strain of *Saccharomyces ellipsoides* (no. 4098 American Type Culture Collection) was also included in the tests.

Procedure

The general method followed was similar to that of Williams *et al.* [1940]. In these experiments, however, the amino-acid samples were added in test tubes in a volume of 1 ml. to which were added 3 ml. of medium containing the appropriate seeding and nutrient factor supplement. After 15 hr. at 30° the cultures were diluted with 5 ml. of saturated chlorothymol solution and the yeast crops determined by the thermocouple method in use in this laboratory [Williams *et al.* 1929].

Results

The results from several experiments are summarized in Table 1. The seedings and control crops are given in mg. per culture, while the effects of the amino-acids are given as percentage increase over the control crop. For the sake of brevity, only the individual amino-acids giving greatest stimulation are included. No more than a trace of toxicity was observed in any case. A maximum stimulating effect was usually obtained with the 0·5 mg. dosage of the individual amino-acids, but the 5·0 mg. dosage of the amino-acid mixtures usually caused the greatest response.

[1] This was 75% pure biotin methyl ester obtained through the generosity of Prof. F. Kögl.

Table 1. *Effects of amino-acids on yeasts*

Yeast strain and seeding (moist) F.B. (0·01 mg.)

Control crop	1·80 mg.
0·5 mg. mixture	+ 53%
5·0 mg. mixture	+170%
0·5 mg. casein digest	+ 43%
5·0 mg. casein digest	+120%

Amino-acids showing greatest individual effects: (56–18%) glutamic acid, asparagine, aspartic acid, leucine, lysine, arginine.

No. 2190 (Narayanan) (0·06 mg.)

Control crop	0·50 mg.
0·5 mg. mixture	+ 500%
5·0 mg. mixture	+1200%
0·5 mg. casein digest	+ 420%
5·0 mg. casein digest	+1000%

Amino-acids showing greatest individual effects: (530–30%) aspartic acid, glutamic acid, asparagine, leucine, serine, methionine, glycine, histidine, tryptophan, arginine, lysine, valine, phenylalanine, cystine, alanine, hydroxyproline, tyrosine.

G.M. (0·01 mg.)

Control crop	1·53 mg.
0·5 mg. mixture	+110%
5·0 mg. mixture	+240%
0·5 mg. casein digest	+ 90%
5·0 mg. casein digest	+170%

Amino-acids showing greatest individual effects: (150–32%) glutamic acid, aspartic acid, arginine, proline, asparagine, phenylalanine, serine, tryptophan, leucine, histidine, glycine, lysine.

H. 1 (0·03 mg.)

Control crop	1·6 mg.
0·5 mg. mixture	+104%
5·0 mg. mixture	+170%
0·5 mg. casein digest	+ 93%
5·0 mg. casein digest	+116%

Amino-acids showing greatest individual effects: (81–32%) glutamic acid, arginine, aspartic acid, asparagine, histidine, proline, cystine, leucine, phenylalanine, serine, tryptophan, tyrosine.

O.P. (0·02 mg.)

Control crop	1·35 mg.
0·5 mg. mixture	+ 91%
5·0 mg. mixture	+290%
0·5 mg. casein digest	+110%
5·0 mg. casein digest	+190%

Amino-acids showing greatest individual effects: (84–15%) glutamic acid, aspartic acid, arginine, asparagine, histidine, leucine, lysine.

S. ellipsoides, No. 4098 (0·01 mg.)

Control crop	0·65 mg.
0·5 mg. mixture	+200%
5·0 mg. mixture	+250%
0·5 mg. casein digest	+180%
5·0 mg. casein digest	+280%

Amino-acids showing greatest individual effects: (130–35%) glutamic acid, asparagine, aspartic acid, leucine, arginine, serine, phenylalanine, proline, alanine.

Discussion

A study of the table reveals a similarity in the various strains of yeast in that all six are considerably affected by five of the amino-acids; glutamic acid, asparagine, aspartic acid, arginine and leucine. Serine, lysine, histidine and

phenylalanine are effective on four organisms, and tryptophan and proline show considerable stimulation on three out of the six strains. In all cases the mixture and the casein digest show marked stimulating actions far above those of individual amino-acids.

In addition to the above similarities between the various strains, marked differences occur. The number of amino-acids that give marked stimulation on each yeast are as follows: No. 2190, 17; H. 1, 12; G.M., 12; No. 4098, 9; O.P., 7; and F.B., 6. Parallel with this there are differences in the extent to which individual amino-acids and amino-acid mixtures are capable of increasing growth, and the maximum effect of the amino-acids in turn depends in part upon the extent to which the control cultures grow. Thus, in the two extreme cases, yeast No. 2190 was affectedby seventeen amino-acids, and the crop was increased 530 % by an individual amino-acid (aspartic acid) and 1400 % by a casein digest. Its multiplication in the control cultures was less than ten-fold. "F.B." yeast, on the other hand, was markedly affected by only six amino-acids; the maximum effect of a single amino-acid was 56 % and the greatest effect of a mixture was 170 %. The control cultures, however, multiplied 180-fold during the incubation period. It appears, therefore, that there is a considerable variability in the amino-acid requirements and the synthetic abilities of various strains of yeast.

All the yeasts which we have studied extensively are capable of synthesizing amino-acids from ammonia-nitrogen; nevertheless, growth is greatly increased when amino-acids are furnished in the culture medium along with the proper nutrient factors. Without these factors we have in general observed amino-acid mixtures, such as represented by a casein digest, to be at least slightly inhibitory. It appears that the amino-acids function in yeast nutrition as does arginine in animal nutrition. The latter is not an obligatory requirement, but growth is increased when it is furnished [Rose, 1938].

It appears probable that amino-acid combinations contribute to an appreciable extent to "bios" activity. In the cases where marked stimulation occurred at the 500 μg. level, as indicated in the above table, a measurable stimulation also occurred down to 50 μg. dosages or less. The designation of "bios" fractions by numbers and letters without definite evidence that each represents an individual compound is unwarranted and the influence of amino-acids in yeast nutrition cannot be overlooked in future "bios" studies.

Experiments with certain strains of yeast not reported here in detail indicated that the nutritional interrelationships between the different amino-acids are complex and may compare strikingly with those observed in this laboratory in connexion with the nutrition of the mould *Trichophyton interdigitale* [Mosher *et al.* 1936].

Summary

The effects of twenty individual amino-acids, a mixture of the same, and a casein digest on five strains of *Saccharomyces cerevisiae* and one strain of *S. ellipsoides* have been studied with the following conclusions:

1. All six strains of yeast were markedly stimulated by the addition of individual amino-acids and of mixtures when the control medium contained an abundant supply of nutrient factors. These amino-acids are not required for growth but growth is enhanced when they are furnished.

2. The five most important amino-acids were glutamic acid, aspartic acid, asparagine, arginine and leucine. These were strongly stimulating on all of the yeast strains tested.

3. A distinct supplementary effect was shown by a combination of all twenty amino-acids.

4. Amino-acids may, depending on the test conditions and the yeast strain used, contribute materially to the "bios" effects of tissue extracts.

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182. THE EFFECT OF ULTRAVIOLET LIGHT ON LIVING YEAST CELLS

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(*Received 11 October 1940*)

In recent communications, Loofbourow *et al.* [1938; 1939; 1940] state that living yeast cells, injured by ultraviolet light, liberate, possibly as a specific response to injury, substances which powerfully stimulate the growth of yeast. These substances they call "proliferation-promoting intercellular hormones" or wound hormones. They suggest, mainly on the basis of spectrographic evidence, that such substances are adenine-guanine nucleotide complexes.

Similar substances with the power of stimulating yeast growth were also obtained by ultraviolet irradiation of living cells of newts and of chick and rat embryos. Such substances from chick embryos are claimed to accelerate the growth of chick fibroblasts *in vitro*.

Loofbourow's conclusions concerning the chemical nature of the growth-promoting substances are based mainly on qualitative tests and on spectrographic evidence. It therefore seems desirable to carry out quantitative chemical analyses of the products released by yeast cells injured by ultraviolet irradiation.

150 g. fresh D.C.L. bakers' yeast (*Saccharomyces cerevisiae*) were washed twice on the centrifuge with 0·9 % NaCl solution and were then suspended in 750 ml. of the same solution. The suspension was divided into three equal portions. One portion (*a*) was autoclaved at 120° for 20 min. Such treatment destroyed cell structure. The second portion (*b*) was exposed for 6 hr. at 13 cm. distance to ultraviolet light from a quartz mercury vapour lamp (3·5 amp., burner volts 150) by being repeatedly run through the rack of quartz tubes described by Stiven [1930] at such a rate that the temperature of the suspension never exceeded 40°. A third portion (*c*) was kept for the same time and at the same temperature as a control but without exposure to ultraviolet light.

The effect of irradiation was followed by withdrawing a drop of the suspension from time to time, treating it with methylene blue and examining microscopically. Living yeast cells are not stained by methylene blue while injured and dead cells stain deeply [Richards, 1932]. At the outset only 1 % of the cells stained, but at the end of irradiation 60 % stained. The cells were shrunken. In the control suspension 10 % of the cells stained at the end of the period. In both cases the total number of cells, counted in a haemocytometer chamber, was unaltered.

The three suspensions were finally centrifuged and the clear supernatant fluid collected. The extract from the autoclaved suspension (*a*) was deep yellow in colour, that from the irradiated cells (*b*) yellow, while the extract from the control (*c*) was colourless. All showed a blue fluorescence in ultraviolet light, the control being only slightly fluorescent. All extracts were kept at 0° and analysed immediately, as they were, especially in the case of the extract from irradiated cells, excellent media for bacterial growth. Extract (*b*), which had *p*H 5·8, gave a positive biuret test but no precipitate on boiling or with nitric acid. It gave a precipitate with tungstic acid. Extract (*c*) from the control

gave a negative response to the biuret test and no precipitate with tungstic acid. Extract (*a*) from autoclaved cells was heavily loaded with protein.

In the three extracts total N was estimated by the micro-Kjeldahl procedure, amino-N by the manometric method of Van Slyke, non-protein-N after deproteinization with tungstic acid, and nucleotide, nucleoside, and free purine-N by the method of Kerr & Blish [1932] as modified by Kerr [1940].

Several separate batches of yeast were treated in this way at different times and the results of a typical experiment are shown in Table 1.

Table 1. *Analyses of centrifuged extracts from autoclaved, irradiated and control cells*

Extract from ...	(*a*) Autoclaved cells mg./100 ml.	(*b*) Irradiated cells mg./100 ml.	(*c*) Control cells mg./100 ml.
Dry wt. (excluding NaCl)	1705	1164	280
Total N	148·4	104·9	12·6
Non-protein-N	72·8	96·2	10·5
Protein-N (by difference)	75·6	8·7	2·1
Amino-N (non-protein)	57·4	63·3	9·0
Nucleotide-N	11·8	11·8	0·4
Nucleoside- and free purine-N	2·9	9·4	1·4

Irradiation sets free a large amount of nitrogenous material. A very small amount is in the form of protein while a high proportion is present as amino-N. Much more N than in the case of the control is present in the form of nucleotide, nucleoside and purine: Table 1 (*b*).

Complete destruction of the cells yields an extract with much protein. The non-protein-N is chiefly amino-N: Table 1 (*a*).

After treating the extract from irradiated cells with 10 % sulphuric acid to hydrolyse nucleic acid derivatives after the method of Jones [1920], adenine was isolated both as the picrate and the sulphate. Small amounts of guanine were also found. Thus it is probable that the nucleotide and nucleoside fractions are chiefly adenine derivatives with small amounts of guanine present.

The growth-promoting powers of the three extracts were tested by seeding Reader's medium [Reader, 1927] with a dilute suspension of yeast cells. Varying amounts of the extracts were added to the media and the cultures grown at 21° in Erlenmeyer flasks as recommended by Williams & Saunders [1934] and by Narayanan [1930]. The crop was estimated after 20 hr. by counting the cells in a haemocytometer. At all concentrations extract (*b*) from irradiated cells had six times the growth-promoting power of the extract from the control. The irradiated suspension had six times as many cells injured as the control, suggesting a correlation between cell injury and liberation of growth-promoting material. The extract (*a*) from autoclaved cells stimulated growth as powerfully as extract (*b*) but, since the extract from autoclaved cells contained more solid matter, weight for weight the material from the irradiated cells was more potent.

Extracts from irradiated and autoclaved cells had greater growth-promoting power than either inositol or aneurin.

Discussion

A large number of substances can accelerate yeast growth, for example, such components of the bios group as inositol, aneurin, biotin, pantothenic acid and adermin. Other substances with similar properties are found in liver extracts

[Williams *et al.* 1940]. Amino-acids such as leucine [Miller, 1936], alanine, aspartic acid, glutamic acid, lysine and arginine stimulate yeast growth, and mixtures of these substances have a more pronounced effect than equivalent amounts of the acids alone [Nielsen & Hartelius, 1938; 1939]. Lysine and arginine are known to be plentiful in the proteins of yeast [Kraut & Schlottmann, 1937].

Such being the case, it is not surprising that rupture of yeast cells by autoclaving should liberate substances with growth-promoting properties towards yeast. Irradiation of yeast, however, yields a surprisingly potent extract without destruction of the cells. This extract has even more non-protein nitrogenous matter than the extract from autoclaved cells. The slow death of the yeast cell does seem to cause liberation of substances into the surrounding medium but it is impossible to say whether or not this is a specific response to injury and an attempt to compensate for the death of some cells by accelerated growth of others.

Among the nitrogenous materials are nucleotides and nucleosides containing adenine and it is highly probable that their presence accounts for the spectrographic findings of Loofbourow *et al.* But it seems premature to conclude that these adenine nucleotide complexes are necessarily the active principles in the extract from irradiated yeast which are responsible for stimulating the growth of yeast.

SUMMARY

Exposure of living yeast cells to ultraviolet light results, without disintegration of the cells, in the liberation of large amounts of nitrogenous material into the surrounding medium. This material has the property of stimulating yeast growth to a marked extent. Although the medium contains adenine nucleotide derivatives, these are not necessarily the growth-promoting principles.

I should like to express my thanks to Prof. R. C. Garry for his helpful encouragement, and to Mr B. L. Andrew and Mr J. D. Findlay for assistance in the analyses.

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183. THE PROTEINASES OF *CLOSTRIDIUM HISTOLYTICUM*

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(*Received 17 October 1940*)

THERE is some disagreement in the literature concerning the proteinases of *Cl. histolyticum*. Kocholaty *et al.* [1938] have claimed that this organism secretes only one proteinase into the medium, this proteinase being extracellular and strongly activated by cysteine. Maschmann [1938], on the other hand, has found results indicating that *Cl. histolyticum* secretes one or more extracellular proteinases which are not activated by cysteine, and that only during later stages of growth of the culture is a clupein-splitting enzyme secreted which is activated by cysteine, and which is presumably intracellular. Maschmann ascribes the cysteine activation of the filtrates of Kocholaty *et al.* to the presence of this clupein-splitting enzyme, but Kocholaty *et al.* have shown that their filtrates are strongly activated by cysteine from the time of the first appearance of the proteinase.

In the work here reported results were found which probably explain this disagreement. It was observed that a strain of *Cl. histolyticum* at first produced filtrates the properties of which were similar to those of Kocholaty *et al.* After repeated sub-cultivation of the strain on cooked meat cultures, in a tryptic digest of casein the organism began to secrete into the medium a system of proteinases, the properties of which were similar to those described by Maschmann.

Methods

Bacterial filtrates. The medium used was the modification of the tryptic digest of casein of Cole & Onslow [1916] as described by Gladstone & Fildes [1940]. In this medium the addition of yeast extract, cysteine or 2·5 % Difco proteose peptone did not bring about an increase in the yield of proteinase. 15 ml. inoculations of 24 hr. cultures of *Cl. histolyticum* (Strain 503, National Collection of Type Cultures) in meat broth tubes were made into 2·5 l. quantities of the medium in 3 l. round-bottomed flasks. After inoculation the flasks were evacuated to 30 mm. Hg, filled with H_2 and incubated at 37° for 16 hr. Cultures grown in this way produced as much proteinase as cultures incubated in a Fildes and McIntosh anaerobic jar. After incubation the cultures, *p*H *c.* 7·2, were clarified by adding about 15 g. kieselguhr per l. and filtering through Whatman no. 1 filter paper with suction. This filtration, which occupies only a few minutes, gave completely clear filtrates the proteinase activity of which was the same as those filtered through Seitz filters.

Estimation of proteinase activity. Since it was necessary to measure true proteinase activity, as distinct from proteolytic activity, and since filtrates of *Cl. histolyticum* cultures are known to contain poly- and di-peptidases, methods which involve the estimation of increase in free amino groups were excluded. In the first stages of this work the following method for estimating proteinase activity was used: 2 ml. of a neutral solution of Hammersten's casein containing 75 mg. casein per ml.; 2 ml. of McIlvaine's buffer, *p*H 7·0; 2 ml. of water, or of a solution of activator or inhibitor; and 2 ml. of the enzyme solution were pipetted into 12 ml. tared centrifuge tubes. The mixtures were then incubated at 37° for 30 min., after which the undigested casein was precipitated by the

addition of 2 ml. 30 % trichloroacetic acid. The precipitate was then centrifuged, resuspended in 10 ml. of 0·5 % acetic acid, and again spun down. After being dried for 1 hr. at 115° the tubes were weighed. The decrease in weight of the casein precipitate, as compared with a control with boiled enzyme, gave a measure of the amount of casein digested by the enzyme. Although some results obtained by this method are recorded, it was later abandoned because of the difficulty experienced in drying the precipitates to constant weight. A more satisfactory method was found to consist in the estimation of non-protein-N formed during digestion. 4 ml. of á 2·5 % solution of casein + 1 ml. of water, or of a solution of activator or inhibitor, were warmed to 40° in a boiling tube and then 1 ml. of the enzyme solution was added. After 15 min. incubation at 40° the undigested protein was precipitated by the addition of 10 ml. of 0·3 *M* trichloroacetic acid and filtered off. N was determined in aliquots of the filtrates by the micro-Kjeldahl method. Enzyme-N was determined by micro-Kjeldahl estimations on aliquots of the dialysed enzyme solutions.

Since these (and all other) methods for estimating proteinase activity cannot be specific for a particular proteinase, and therefore measure *total* proteinase activity, the term "the proteinase" should be read as "the proteinase or proteinases" throughout this paper.

Results

When this work was first started the *Cl. histolyticum* culture filtrates invariably contained a proteinase, the activity of which was increased by about 50 % by the addition of cysteine. This cysteine activation was quantitatively the

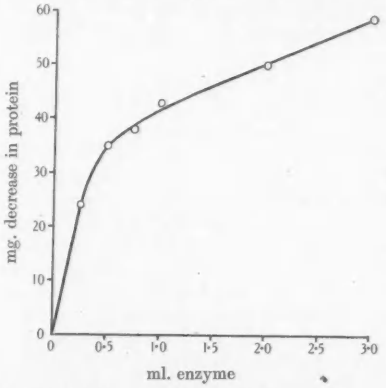

Fig. 1. Activity curve for crude filtrate. 150 mg. substrate, 3 hr. digestion.

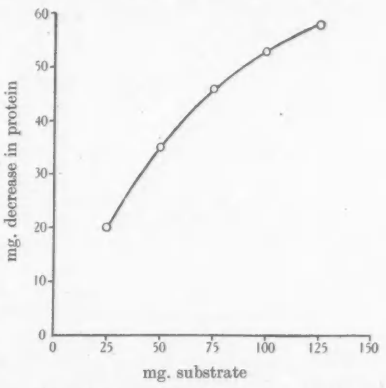

Fig. 2. Influence of substrate concentration on digestion by crude filtrate. 2 ml. filtrate, 3 hr. digestion.

same, whether the cultures were filtered after 8, 12 or 16 hr. incubation. The activation could be further increased in the presence of *M*/150 $FeSO_4$. Typical figures for the decrease in trichloroacetic acid-precipitable casein after 4 hr. digestion by the crude filtrates are:

2 ml. filtrate alone	43 mg.
2 ml. filtrate, *M*/150 cysteine	69 mg.
2 ml. filtrate, *M*/150 cysteine, *M*/150 $FeSO_4$ ...	87 mg.

These findings are in agreement with those of Kocholaty *et al.*

The activity curve and a curve showing the effect of substrate concentration for the crude filtrate are given in Figs. 1 and 2.

The filtrates obtained with the medium used contained very little protein and consequently it was impossible to obtain quantitative recovery of the proteinase by fully saturating the filtrates with ammonium sulphate. It was found, however, that the filtrates could be concentrated tenfold by distillation at 15 mm. pressure and about 15° internal temperature without appreciable loss of activity. From dialysed concentrates prepared in this way the proteinase could be precipitated by the addition of two volumes of methyl alcohol and thus recovered with yields of about 90 %. The precipitates could be dried, but with a loss of about 40 % of their activity. The dried powders retained their activity, without loss, for at least 3 months. The specific activity of the proteinase (mg. protein decrease per mg. enzyme N in 30 min.) was increased about fourfold by precipitation with methyl alcohol, and the activity of the precipitates was increased by cysteine to the same extent as the crude filtrates, as can be seen from the following typical figures:

	Specific activity	
	Without cysteine	With cysteine
Crude filtrate	65	91
Methyl alcohol precipitate	273	370
Dried precipitate	164	230

Fig. 3 shows the time curves for the partially purified proteinase. It will be seen that the increase in free NH_2-groups continues after the decrease in protein has stopped. This might be due to further action of the proteinase on the partially split protein, or to the action of poly- and di-peptidases.

Attempts to increase the specific activity of the proteinase by further fractional precipitation with organic solvents or with ammonium sulphate were not successful. It was found that the proteinase could be quantitatively adsorbed from a solution containing the methyl alcohol powder (10 mg./ml.) by kaolin (4 mg./ml.) at *p*H 5. A number of eluents was tried, and borate buffer at *p*H 9 was found to be the most effective. With this eluent a preparation was made which was inactive unless cysteine was added. Not more than about 30 % of the original activity could be recovered by this process of adsorption and elution. Furthermore, the proteinase could not be concentrated by the elution since it was found that when the volume of the eluent was smaller than the volume from which the proteinase had been adsorbed the recovery was correspondingly lower; nor was it possible to concentrate the enzyme by adsorbing from a more concentrated solution. The specific activity of the eluted proteinase (mg. protein decrease per mg. enzyme N in 30 min.) was 835, a ninefold increase with respect to the cysteine-activated crude filtrate. The method of adsorption and elution was not suitable as a preparative procedure, and further purification of the eluted proteinase was not possible. Its chief interest lies in the fact that the preparation was entirely inactive in the absence of cysteine. It was also found that *M*/100 cyanide could activate this preparation. Dialysis of the system proteinase-reducing agent caused the activity to disappear, but it could be restored by the addition of cyanide or cysteine.

Variation in the strain. After the strain of *Cl. histolyticum* had been maintained for about 3 months by weekly sub-cultivation from meat tubes the cultures of the organism began to show diminishing proteinase activity, without apparent decrease in bacterial growth. Within 2 weeks of this diminution being

observed the cultures acquired a very strong odour and the filtrates became completely inactive. Examination showed that the cultures were not contaminated, but it was noticed that tube cultures of the organism grew equally well aerobically and anaerobically. The organism was plated out on broth-agar and incubated for 48 hr. in an anaerobic jar. After incubation 6 colonies were picked off and each was sown into a separate meat tube. After 48 hr. incubation in the anaerobic jar inoculations from each of the meat tubes were made into two series of broth tubes. One of these series was incubated anaerobically, and the other aerobically, for 48 hr. At the end of incubation it was observed that

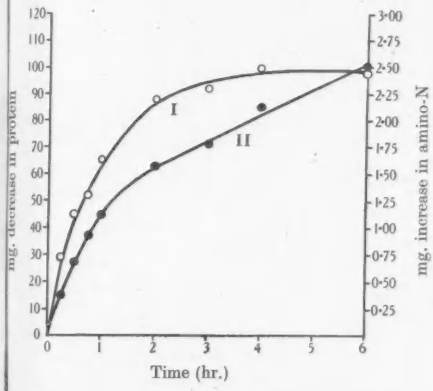

Fig. 3. Time curves for partially purified preparation. I, decrease in protein precipitable by trichloroacetic acid. II, increase in free NH_2-groups (Van Slyke manometric method, 5 min. with HNO_2).

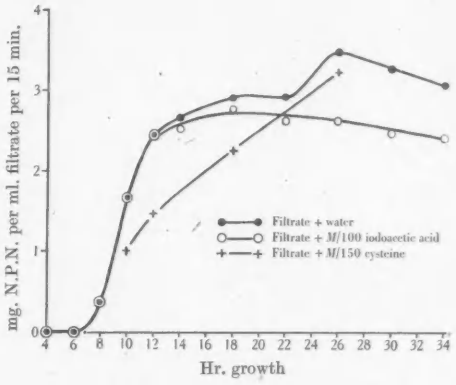

Fig. 4. Production of extra- and intra-cellular proteinases during growth of culture.

there was good growth in all the anaerobic tubes and in three of the aerobic tubes. The other aerobic tubes showed no growth. One of the cultures which grew anaerobically, but not aerobically, was then chosen for further cultivation. Inoculations were made from the appropriate meat tube into 12 tubes containing alkaline egg medium, which were then sealed and stored. When in the later stages of the work it was found that the activity of the cultures was diminishing the organism was recultivated from the spores in one of these tubes. When these new cultures were inoculated into 2·5 l. quantities of the tryptic digest of casein a good, odourless growth was obtained after 16 hr. incubation. The activity of the filtrates was now about 50% greater than the activity of the previous filtrates, but, on the other hand, this activity could not be increased by the addition of cysteine or cyanide. The relation between the new and old cultures can be seen from the following table:

	mg. protein decrease per 2 ml. filtrate in 30 min.		
	Without cysteine	With cysteine	Odour
Old cultures	20	30	Slight
Inactive cultures	0	0	Very strong
New cultures	30	30	None

With the new filtrates it was again possible to prepare methyl alcohol precipitates from dialysed concentrates, and the proteinase could be quantitatively adsorbed by kaolin from solutions of these precipitates. Borate buffer, *p*H 9, again proved to be the most effective eluent, and the recovery of activity was again about 30%. The proteinase in the eluates had a very low but definite activity in the absence of cysteine, but in the presence of cysteine the activity was increased fivefold. All proteinase estimations given below were done by determining the increase in non-protein-N. The specific activity (mg. non-protein-N formed per mg. enzyme N in 15 min.) of the cysteine-activated eluates was 580. The proteinase could not be concentrated in the eluates, and consequently a further increase in the specific activity of the proteinase could not be effected. When the eluate + *M*/150 cysteine was incubated with *M*/100 iodoacetic acid the activity was reduced to one-third, but never quite to the level of the eluate alone. It was then found that the activity of the methyl alcohol precipitates was reduced by about 30% by *M*/100 iodoacetic acid. The amount of activity in the borate eluates which was due to cysteine corresponded to the loss of activity in the methyl alcohol precipitates due to iodoacetic acid. Typical figures for equivalent amounts of enzyme are given below:

Enzyme preparation	mg. N.P.N. formed in 15 min.
Methyl alcohol precipitate	3·07
Do + *M*/150 cysteine	3·00
Do + *M*/100 iodoacetic acid	2·18
Borate eluate	0·25
Do + *M*/150 cysteine	1·36
Do + *M*/150 cysteine + *M*/100 iodoacetic acid	0·43

These figures indicate that the methyl alcohol precipitate consisted of at least two proteinases, of which one was not activated by cysteine and not inhibited by iodoacetic acid, and the other was activated by cysteine and inhibited by iodoacetic acid. It appeared that both these enzymes were adsorbed by kaolin, and that one of them was quantitatively and nearly specifically eluted by borate buffer at *p*H 9.

In order to follow the secretion of these enzymes during the growth of the culture the following experiment was carried out: 1·5 l. of the inoculated medium were placed in a 2 l. flask which was kept at 37°. Sterile H_2 was bubbled through the medium and at regular intervals 10 ml. samples of the culture were withdrawn under sterile conditions. The samples were filtered through Seitz filters and aliquots were incubated with water, *M*/100 iodoacetic acid and *M*/150 cysteine respectively for 30 min. before being tested for proteinase activity. The results are plotted in Fig. 4. It will be seen that up to the 12th hr. of growth the proteinase which appeared in the medium was neither inhibited by iodoacetic acid nor activated by cysteine; it was, in fact, quite strongly inhibited by cysteine. After 12 hr. another proteinase appeared in the medium. This proteinase was inhibited by iodoacetic acid and activated by cysteine, and was presumably an intracellular enzyme. These findings are in agreement with those of Maschmann [1938]. The fact that a 16 hr. filtrate contained one set of enzymes that was inhibited by cysteine, and another that was activated by cysteine, explains why cysteine had no apparent effect on the crude filtrates.

The intracellular proteinase could be separated to a fair degree from the mixture of intra- and extra-cellular proteinases by adsorption on kaolin, followed by elution with borate buffer, as already described. The extracellular proteinase could be obtained free from the intracellular proteinase by filtering the culture after 8 hr. growth, i.e. before the appearance of the intracellular proteinase in

the medium. The specific activity of the extracellular proteinase in the crude 8 hr. filtrates was 19·4 (mg. non-protein-N formed per mg. enzyme-N in 15 min.). After concentration, precipitation with fully saturated ammonium sulphate and precipitation with 66% methyl alcohol a preparation was obtained with a specific activity of 86, but further attempts at purification were unsuccessful. This preparation was unaffected by iodoacetic acid and inhibited about 40% by cysteine.

Discussion

These results show that strains of *Cl. histolyticum* on continued sub-cultivation vary in properties when picked off as a single colony. The proportion of proteolytic enzymes is only one among the properties which vary in this way, and this provides an explanation for the apparent contradictions of previous workers in this field.

Summary

It was found that a strain of *Cl. histolyticum* produced an extracellular proteinase which was activated by cysteine. After continued sub-cultivation the strain was plated out and cultures from a single colony produced an extracellular proteinase which was inhibited by cysteine and an intracellular proteinase which was activated by cysteine.

The author is grateful to Drs Marjory Stephenson, Muriel Robertson and Malcolm Dixon for much helpful advice, and to the Royal Commissioners for the Exhibition of 1851 for a Senior Studentship.

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184. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

68. SYNTHESIS OF CYNODONTIN (1:4:5:8-TETRAHYDROXY-2-METHYLANTHRAQUINONE) A METABOLIC PRODUCT OF SPECIES OF *HELMINTHOSPORIUM*

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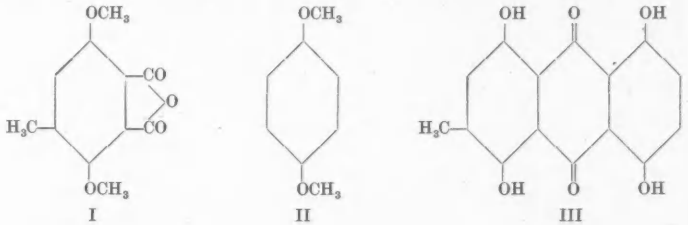
(*Received 1 November 1940*)

Cynodontin was first isolated by chloroform extraction of the dried mycelium of laboratory cultures of the fungi *Helminthosporium cynodontis* Marignoni and *H. euchlaenae* Zimmermann by Raistrick *et al.* [1933, 1] and by the same workers from *H. avenae* Eidam [1934]. It was shown to be almost certainly 1:4:5:8-tetrahydroxy-2-methylanthraquinone (III) since it was obtained *in vitro* [Raistrick *et al.* 1933, 1], by the oxidation with manganese dioxide and concentrated H_2SO_4 of helminthosporin, a metabolic product of *H. gramineum* Rabenhorst [Charles *et al.* 1933] and helminthosporin was shown by synthesis to be 4:5:8-trihydroxy-2-methylanthraquinone [Raistrick *et al.* 1933, 2].

We have now confirmed by synthesis the suggested molecular structure for cynodontin. 4-Methyl-3:6-dimethoxyphthalic anhydride (I) [Anslow & Raistrick, 1940] was condensed with 1:4-dimethoxybenzene (II) by treatment with anhydrous $AlCl_3$ in CS_2 solution. Simultaneous demethylation and ring closure of the resulting acid was effected by heating with conc. H_2SO_4 at 150°. The resulting polyhydroxyanthraquinone which must, from its method of synthesis, be 1:4:5:8-tetrahydroxy-2-methylanthraquinone (III) was identical in all its properties with cynodontin isolated from different species of *Helminthosporium*.



Experimental

(*a*) *Condensation of 4-methyl-3:6-dimethoxyphthalic anhydride with 1:4-dimethoxybenzene.* Freshly powdered anhydrous aluminium chloride (4·4 g.) was added at short intervals in five approximately equal amounts to a mixture of 4-methyl-3:6-dimethoxyphthalic anhydride (2 g.) and 1:4-dimethoxybenzene (4 g.) in dry carbon disulphide (50 ml.). The mixture, which quickly became

yellow and then orange in colour, was boiled under reflux for 8 hr. After standing overnight the supernatant CS_2 was decanted from the dark brown semi-solid mass which was decomposed with 2 *N* HCl (75 ml.), the mixture being cooled during the process. The residue remaining after evaporation of the CS_2 solution was added and the excess of 1:4-dimethoxybenzene was removed by distillation in steam. The oily residue quickly solidified on cooling and was extracted with ether (1 l.). The ether solution was dried over anhydrous $MgSO_4$ and on removal of the solvent the crude benzoyl acid was obtained as a brownish yellow oil which later solidified (2·44 g.).

(*b*) *Demethylation and ring closure of the substituted benzoyl acid.* The crude acid (2·44 g.), without further purification, was heated with conc. H_2SO_4 (20 ml.) in an oil bath at 150° for 35 min. The H_2SO_4 solution, which was initially greenish-blue in colour with a fine green fluorescence, became deep blue in colour with an intense red fluorescence after 15 min. heating. The anthraquinone, which was separated by pouring the cooled reaction mixture into iced water (600 ml.), was coagulated by heating on a boiling water bath for 30 min. and was separated by filtration as a chocolate brown amorphous powder (1·40 g.). This crude material was extracted with chloroform (Soxhlet) and there separated from the eosin-coloured extract brown leaflets with a bronze lustre (0·76 g.; M.P. 255–260°). Evaporation of the chloroform mother liquors and sublimation of the residue in a high vacuum gave a further 0·36 g. of crude anthraquinone.

(*c*) *1:4:5:8-Tetraacetoxy-2-methylanthraquinone* (*cynodontin tetraacetate*). The anthraquinone (0·76 g.; M.P. 255–260°) separating from chloroform was purified by conversion into the acetate. It was dissolved in a mixture of pyridine (25 ml.) and acetic anhydride (5 ml.) and held at 37° for 2 days. The reaction mixture was filtered and the filtrate was poured into ice-water (400 ml.). The acetate (0·87 g.) which separated was crystallized twice from glacial acetic acid (norite). Pale yellow prisms (0·44 g.), M.P. 224–226° alone or in admixture with the tetraacetate prepared from natural cynodontin. (Found: C, 60·57, 60·42; H, 3·99, 4·12 %. $C_{23}H_{18}O_{10}$ requires C, 60·77; H, 4·00 %.) A further 0·11 g. of pure acetate was obtained by acetylating in the same way the 0·36 g. of crude anthraquinone obtained from the chloroform mother liquors.

(*d*) *1:4:5:8-Tetrahydroxy-2-methylanthraquinone* (*cynodontin*). Pure 1:4:5:8-tetraacetoxy-2-methylanthraquinone (0·4 g.) was hydrolysed by heating under reflux on a boiling water bath with 2 *N* aqueous NaOH (30 ml.) in an atmosphere of nitrogen for 2 hr. The tetraacetate quickly dissolved to give an intensely blue-violet solution. The anthraquinone was precipitated with 2 *N* HCl (40 ml.), coagulated by heating on a boiling water bath for 30 min., filtered, washed and dried. This material (0·25 g.) was crystallized from pyridine (30 ml. with norite) and 1:4:5:8-tetrahydroxy-2-methylanthraquinone was thus obtained in brown leaflets with a fine bronze lustre (0·19 g.; M.P. 260–261°), alone or in admixture with cynodontin from *Helminthosporium cynodontis*. (Found: C, 62·94, 62·88; H, 3·67, 3·65 %. $C_{15}H_{10}O_6$ requires C, 62·91; H, 3·52 %.) The synthetic and natural specimens are both insoluble in cold dilute aqueous Na_2CO_3. Both dissolve in cold aqueous 2 *N* KOH to give deep blue-violet solutions and solutions of both in cold conc. H_2SO_4 are intense pure blue in colour with a fine red fluorescence.

Summary

Cynodontin (1:4:5:8-tetrahydroxy-2-methylanthraquinone), a metabolic product of different species of *Helminthosporium*, has been synthesized. 4-Methyl-3:6-dimethoxyphthalic anhydride was condensed with 1:4-dimethoxybenzene

and the resulting acid was simultaneously demethylated and converted into 1:4:5:8-tetrahydroxy-2-methylanthraquinone by heating with conc. H_2SO_4. The synthetic and natural products were shown to be identical.

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185. TISSUE PROTEINS OF SOME CRYPTOGAMS: THE AMIDE, TYROSINE AND TRYPTOPHAN CONTENTS

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(*Received 26 October 1940*)

From the standpoint of biological evolution it might be conjectured that some modification in the amino-acid compositions of tissue proteins would accompany evolutionary development, but it might be argued contrariwise that a pronounced change in composition could be anticipated only in association with a change in function of a tissue. Correspondingly, among the present day descendants of a common ancestor, according to their degrees of development from, or retention of, the primitive characteristics, one might expect variation in the protein composition or anticipate constancy.

Extensive analyses of animal proteins have been published and although many of them require repetition by newer and more critically examined methods, certain regularities have been established soundly enough, e.g. the high cystine contents of the proteins of epidermal structures like hair, wool, horn etc., and the high arginine contents of the protamines. Block & Vickery [1931], and Block [1934] and in subsequent articles, have drawn attention to the constancy of the ratio of the histidine, lysine and arginine contents of proteins in groups arranged according to biological origin, physical properties etc. The results of Bailey's [1937] partial analyses of muscle proteins may be expressed briefly as follows: such evolutionary developments as can be traced through the crustaceans, fish, birds and mammals have probably been without marked influence upon the compositions of the skeletal muscle myosins. It would seem, in general, that the proteins of animal tissues having similar functions will have similar physical properties and will be similar in composition.

Comparative studies of plant proteins have generally been confined to the seeds of the angiosperms, chiefly on account of the ease with which the proteins could be obtained reasonably free from other classes of organic material. The more recent analyses have sustained the conclusions drawn from the earlier work, that between seed proteins the differences in composition are frequently very large but that marked regularities do exist, as in the high proline and glutamic acid contents of the gliadins of seeds of the Gramineae.

It is only in recent years that detailed knowledge of the compositions of proteins of the leaves of these higher plants has been acquired. As reviewed by Chibnall [1939], the analyses show remarkable uniformity of composition, almost comparable with the degree of uniformity among mammalian myosins. There are differences as between the Leguminosae, Gramineae etc. but they are not large.

The main purpose of this article is to record partial analyses of the proteins of somatic, photosynthesizing parts of some lower land plants, and to compare

the results with those for the higher plants. Results obtained with *Trifolium subterraneum*, a legume of considerable economic importance in Australia, are included.

Preparations

Freshly harvested plant tissues were used. The thalli of *Lunularia cruciata* were freed from rhizoids and gemmae. The pinnae of *Pteridium aquilinum* were stripped from the petioles and were selected free from sori. The leaf and stem material of *Selaginella* sp. (probably Jungermannioides Group) was free from stroboli and root-bearing branches. Juices from crushed samples of these tissues were found to be of *p*H 6·0, 5·2 and 5·5 respectively. Only the leaves of *Trifolium subterraneum* were macerated for extraction of protein, the fresh-weight ratio of leaves to petioles was 1 : 1·2, and the dry-weight ratio was 1 : 0·7.

The protein preparations are listed in Table 1 in the order, from top to bottom, of increasing evolutionary development of the plants. In the order of the columns are shown: the label of the protein preparation, viz. a number

Table 1. *The plant materials and protein preparations*

Prep.	Source and date	% dry wt.	% N in dry tissue (*t*, *c*, *p*)	% extracted or residual N (*t*, *c*, *p*)	% N in prep.	% ash in prep.
1W	*Lunularia cruciata* [thalli] x. 39–vi. 40 (liverwort)	7·1	3·85*t*, 2·41*c*, 2·27*p*	—	3·92	3·5
2W	*Pteridium aquilinum* [pinnae] 7. vi. 39 (bracken)	18·7	4·32*t*, 3·80*c*, 3·69*p*	—	5·82	1·6
2E	Do.	—	—	69·9*t*, 69·9*c*, 69·3*p*	11·10	1·4
2R	Do.	—	—	30·1*t*, 31·5*c*, 31·5*p*	2·70	1·6
2E1	Do.	—	—	46·5*t*, 42·1*c*, 41·8*p*	12·00	1·3
3W	*Selaginella* sp. [leaves and stems] 24. x. 39 (smaller club-moss)	13·6	2·32*t*, 1·92*c*, 1·82*p*	—	3·12	9·9
4W	*Trifolium subterraneum* [leaves] 10. xi. 39 (subterranean clover)	18·8	5·10*t*, 4·26*c*, 4·12*p*	—	7·17	0·7
4E	Do.	—	—	38·7*t*, 34·4*c*, 33·3*p*	14·10	0·4
4R	Do.	—	—	61·3*t*, 66·3*c*, 65·8*p*	5·26	3·1
4′W	*Trifolium subterraneum* [petioles] 10. xi. 39 (subterranean clover)	10·8	1·84*t*, 1·38*c*, 1·30*p*	—	2·74	0·7

followed by an abbreviation, W, E or R indicating whether "whole", "extracted" or "residual"; the species of plant and type of tissue, with date of harvest; the percentage dry weight of the tissue; the percentage total (*t*), coagulable (*c*) and protein (*p*) N in the dry tissue; the percentage of the tissue-N (*t*, *c* and *p*) appearing in an extracted juice or tissue residue; the percentage N in the dry protein preparation; and finally, the percentage ash in it by ignition in air at about 600°.

The extracted preparations were made by methods discussed earlier [Lugg, 1939], in which and previous publications [1938, 2, 3], the meanings of terms such as "coagulable N", "protein-N", etc. have been discussed fully.

Preparation 2E was made by macerating the pinnae fairly finely in a power-driven mincer with a borate buffer (*p*H 9), sieving the mash, centrifuging the liquid for a minute or so in a field of 200 × gravity, and flocculating and coagulating the protein by bringing the juice to about *p*H 4·5, adding 0·7 vol. of alcohol and heating to about 75°. Preparation 2E1 was made by treating an aliquot of the unflocculated juice (adjusted to *p*H 9·2) with a cooled mixture of 0·8 vol. of alcohol and 0·2 vol. of ether and filtering the liquid off after 15 min.

through a pad of paper pulp before flocculating and coagulating the protein. The data have been calculated back as if all the juice had been used for each preparation, 2E and 2E1. Preparation 4E was made by coarsely macerating the leaves in the mincer with a *p*H 9 borate buffer to which had been added 0·8 vol. alcohol and 0·2 vol. ether. The mash was sieved and centrifuged at 1000 × gravity for 30 min. before flocculating and coagulating the protein. The coagula were extracted as usual with hot alcohol, hot dilute citric acid solution (or Na citrate at *p*H 3·5), hot alcohol again and finally warm ether. Values recorded in Table 2 have been adjusted for the small errors, one way or the other, to show perfect recovery of tissue total N between residue and juice (except of course, in the case of 2E1 where material was retained by the filter pad). Preparations 1W, 2W, 3W and 4W were made by extracting the non-protein-N from samples of the powdered dry tissues as described by Lugg [1939], and 2R and 4R were obtained from the tissue residues after extraction of 2E and 4E respectively, by removing the non-protein-N.

It was anticipated, from the work with grasses [Lugg, 1939], that 2E1, by virtue of the method of preparation, would be purer than 2E, and its higher N content indicates that this is so. The procedure followed in extracting 4E was suggested [Lugg, 1939], more particularly in association with fine maceration, but has not been used before.

Methods of estimation

Nitrogen estimations were made by the Kjeldahl method. Amide estimations were performed as described previously [Lugg, 1938, 3; Shore *et al.* 1936]. Tyrosine, diiodotyrosine and tryptophan were estimated by Lugg's [1937] methods (modified Millon colour reaction for tyrosine, special colour reaction between nitrous acid and tryptophan mercurial for tryptophan), following alkali and alkali-stannite hydrolyses of the protein preparations as described by Lugg [1938, 1].

Results and discussion

In Table 2 are shown in the order of the columns: the label of the protein preparation, its N content, and the amide, tyrosine and tryptophan contents as percentages of the protein-N appearing in these forms. The random errors, as percentages of the values shown, are believed not to exceed about 0·5, 1·5, 2–3 and 3–4 respectively.

Table 2. *Partial analyses of the proteins*

Prep.	% N	% amide-N	% tyrosine-N	% tryptophan-N
1W	3·92	5·52	2·43	1·70
2W	5·82	5·12	2·06	1·14
2E	11·10	4·97	2·08	1·13
2R	2·70	5·14	2·20	1·13
2E1	12·00	4·92	2·14	1·19
3W	3·12	5·31	2·60	1·43
4W	7·17	5·29	2·53	1·75
4E	14·10	5·35	2·64	1·62
4R	5·26	5·39	2·64	1·67
4′W	2·74	5·91	—	—

Table 3 gives a comparison of tyrosine and tryptophan estimations, following simple alkaline hydrolysis and alkali-stannite hydrolysis. Simple alkaline hydrolysis of these impure protein preparations normally does not provide as clean solutions as alkali-stannite hydrolysis does, and on this account the errors

Table 3. (*Supplement to Table 2*)

	Alkaline hydrolysis		Alkali-stannite hydrolysis	
Prep.	% tyrosine-N	% tryptophan-N	% tyrosine-N	% tryptophan-N
1W	2·36	1·65	2·46	1·72
2E1	2·17	1·13	2·12	1·22
3W	2·53	1·47	2·65	1·40

of estimation tend to be higher in the former cases. Inasmuch as the former permits the estimation of tyrosine and the latter of tyrosine plus diiodotyrosine, the diiodotyrosine content follows by difference, though presumably any dibromotyrosine present would be included. Brand & Kassell [1939] have confirmed the procedure as satisfactory for the estimation of diiodotyrosine. The differences appearing in Table 3 are not considered significant, and the tyrosine and tryptophan contents shown in Table 2 are the weighted means of all estimations.

From the methods of extraction, preparations 2E, 2E1 and 4E were expected to be substantially representative of the whole proteins of the tissues, and the analyses, so far as they go, do not suggest that they were appreciably unrepresentative.

The leaf protein of *Trifolium subterraneum* does not depart, in these analyses, from the ranges of values obtained with the leaf proteins of other legumes. The protein of the petioles proved to be of higher amide content than that of the leaves.

The tyrosine and tryptophan contents of the *Pteridium aquilinum* preparations and the tryptophan content of the *Selaginella* sp. preparation are rather lower than those of the generality of angiosperm leaf proteins; in fact, the tryptophan contents of the *Pteridium aquilinum* preparations are lower than that of any of the angiosperm leaf proteins examined by these methods. However, it may be said that the amide, tyrosine and tryptophan contents of the proteins of the main photosynthesizing tissues of these cryptogams (*Lunularia cruciata* of the bryophytes, and *Pteridium aquilinum* and *Selaginella* sp. of the pteridophytes) are of much the same magnitude as those of the phanerogams examined [Lugg, 1938, 3; 1939; Chibnall, 1939].

It should be noted that the *Lunularia cruciata* preparation came from the gametophyte (with cell nuclei of *n* chromosomes), whereas the other cryptogam and all the phanerogam preparations came from sporophytes (with cell nuclei of *2n* chromosomes).

Summary

Partial analyses of protein preparations from the photosynthesizing tissues of one member of the bryophyte division (*Lunularia cruciata* of the class Hepaticae) and two members of the pteridophyte division (*Pteridium aquilinum* of the class Filicales and *Selaginella* sp. of the class Lycopodiales) have been made. The amide, tyrosine and tryptophan contents as percentage of the protein-N were respectively 5·52, 2·43 and 1·70 for *Lunularia cruciata*, 4·92–5·14, 2·06–2·20 and 1·13–1·19 for *Pteridium aquilinum*, and 5·31, 2·60 and 1·43 for *Selaginella* sp. These are of much the same magnitude as values obtained with leaf proteins of the angiosperms in the spermatophyte division, but the tryptophan contents of the *Pteridium aquilinum* preparations are rather lower than any encountered heretofore.

The amide, tyrosine and tryptophan contents of protein preparations from leaves of *Trifolium subterraneum* were 5·29–5·39, 2·53–2·64 and 1·62–1·75

respectively, as percentage of the protein-N (similar to other legumes), and the amide content of the petiole protein was 5·91.

Thanks are due to Mr R. A. Weller for valuable assistance, and to Prof. J. G. Wood, Miss B. S. Barrien and Miss C. M. Eardley for classification of plant material.

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186. THE ESTIMATION OF PROTHROMBIN IN HUMAN PLASMA

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IT is well known that a deficiency of vitamin K leads to a reduction in plasma prothrombin and a consequent tendency to haemorrhage. Estimations of plasma prothrombin are therefore needed in experimental studies on vitamin K deficiency, and in the assay of the vitamin and related compounds. Moreover, the estimation has an important clinical application, especially in estimating the tendency to haemorrhage in cases of biliary obstruction, and so giving warning of the risks of operation. There is abundant evidence that the tendency to haemorrhage in these cases is due to low plasma prothrombin, which results from deficient absorption of vitamin K in the absence of bile salts from the intestine.

A consideration of the methods available for estimating plasma prothrombin indicated the need for a technique which should combine quantitative accuracy with reasonable simplicity. Indeed it seemed doubtful whether any method could be regarded as truly quantitative. That which seemed most satisfactory in principle was the method first published by Warner *et al.* [1936] and subsequently modified by them [Smith *et al.* 1937]. It is a very elaborate method, and apparently has only been used by its authors. The present paper describes a study of the factors involved in a method of this type, with a view to securing quantitative results, and introduces a modified technique which is distinctly simpler than the original.

The principle of the original method was as follows. The plasma to be tested was freed from fibrinogen by treatment with a thrombin preparation, and the mixture kept until the added thrombin had been completely destroyed by the antithrombin of the plasma. The fibrinogen-free plasma was then suitably diluted with saline, treated with calcium and a tissue extract (prepared from beef lung), and allowed to incubate for the conversion of prothrombin into thrombin. The thrombin activity of the incubating mixture was estimated by determining the time taken to form a clot with added fibrinogen. It was found that during incubation the thrombin first increased, then reached a temporary steady level, and later decreased. The unit of thrombin adopted was the amount giving a clotting time of 15 sec. under the conditions of the method, and the estimation was based on determining the dilution of plasma required to give this standard clotting time during the period of maximal activity. For the plasma of a normal dog, which was the first species studied, the titre obtained was 325–400 units, and the incubation time required for thrombin development was 30–90 sec. Later the method was applied to various species, including man [Warner *et al.* 1939, 1, 2]. Human plasma gave a titre not much lower than that of the dog, but the rate of conversion of prothrombin into thrombin was much slower in human plasma.

Now the method of calculation here used implies that two samples of plasma, normal and abnormal, can be precisely compared on the basis of the different dilutions which give the same thrombin activity—for example, a plasma of "50 units" diluted 1 : 50 is compared with a plasma of "300 units" diluted 1 : 300. This will only be satisfactory if the antithrombin of the plasma is inactive at both dilutions. The authors believed this to be the case; they considered that under the conditions of their method prothrombin was completely converted into thrombin without loss. But some of their statements seem to cast doubt on this point. First, in their incubating mixtures they obtained only a temporary steady level of maximal thrombin activity followed by deterioration. This suggests that the temporary steady level may not represent complete conversion of prothrombin into thrombin but may result from a balance between the rates of formation and destruction of thrombin. If this is so, the antithrombin effect may be greater in the lower dilutions of plasma, and this would lead to an underestimate of the titre of samples of low prothrombin content. Secondly, they state that it is necessary to determine exactly the dilution required to give the standard clotting time; an interpolation curve could be used only to correct for deviations of 1 or 2 sec. from the standard 15 sec. This seems to imply that they obtained no regular relationship between dilution and thrombin activity applicable to all plasma samples. If a regular relationship had been obtained, it should have been possible to determine it for normal plasma over a wide range of dilutions and compare the normal with the abnormal at any convenient dilution. The method of calculation therefore seems to be arbitrary.

From the technical point of view, the method is complicated because it requires two reagents, thrombin and fibrinogen, which are laborious to prepare and which are only stable for short periods. In the new modification, the procedure is simplified in two respects. First, the preliminary removal of fibrinogen from the plasma is omitted, and this eliminates the need for thrombin as a reagent. As the plasma is used in high dilution, the amount of fibrinogen in the mixture incubated with calcium and thromboplastin is very small, and the slight clot which forms does not interfere with the measurement of samples for clotting tests with added fibrinogen. It was found, too, that the presence of this trace of fibrinogen caused no reduction in the thrombin activity developed in the mixture, as compared with samples from which fibrinogen had been removed.

Secondly, in the new modification, there is a perfectly regular relationship between plasma dilution and thrombin developed, and it is therefore possible to base the estimation on a short series of convenient dilutions, instead of discovering the precise dilution of the plasma which gives a standard thrombin activity. The new method differs also in other details. The source of thromboplastin is rabbit brain, chosen as being more easily pulped and extracted than lung. The concentrations of calcium and tissue extract used are those which have been determined to fall within the optimum range for the conversion of prothrombin into thrombin. Both must be in optimal concentration; an excess of either is inhibitory. Incubation for thrombin formation is carried out at 15–20°, because the thrombin is most stable in this range of temperature [Ferguson, 1938, 1, 2], and clotting tests with fibrinogen are made at 37–38°.

The rates of conversion of prothrombin into thrombin are slow. The slow conversion is partly due to the special properties of human prothrombin, and partly to the low incubation temperature.

When due allowance is made for the known differences (in volume relations and temperature) between the modified method and the original method of

Warner *et al.*, it is found that the modified method gives definitely lower activity than the original, but it seems to have the advantage of greater regularity.

It will be convenient to give first the description of the new technique and method of calculation, and then to proceed to a fuller consideration of the different reactions involved and of the grounds for regarding the method as giving quantitative results.

The estimation of plasma prothrombin

Reagents.

(1) Isotonic sodium chloride (0·9 %).

(2) Glyoxaline buffer.

Take 1·72 g. glyoxaline. Add 90 ml. *N*/10 HCl and make up to 100 ml. with water.

This solution is isotonic with blood and gives *p*H 7·25.

(3) Tissue extract.

(*a*) Stock tissue preparation. Take the brain of a freshly killed rabbit. Free it from blood vessels as completely as possible by stripping off the membranes. Grind the tissue in a mortar with acetone. Pour off the liquid and grind the tissue with fresh acetone. Repeat this several times, and then transfer the dry tissue to a Büchner filter and remove the remaining acetone by suction. If necessary, wash the tissue with acetone again and continue suction until the tissue is quite dry. The dry powdered brain is kept as a stock, and retains its activity for about 10 days.

(*b*) For use, an extract of the dried brain is made in a solution containing 0·05 g. anhydrous $CaCl_2$ and 0·855 g. NaCl per 100 ml.

Take 0·05 g. dried brain; grind it in a mortar with 5 ml. of the calcium-containing saline, adding the solution gradually so as to obtain a smooth emulsion. Centrifuge the mixture; take off the supernatant opalescent fluid, and dilute it with three times its volume of the calcium-containing saline. This extract, containing calcium and thromboplastin, is made up fresh as required.

(4) Fibrinogen.

For the preparation the following reagents are needed:

(*a*) Magnesium hydroxide suspension.

Take 100 ml. 20 % $MgCl_2$ and gradually add 25 ml. concentrated ammonia. Filter on a Büchner funnel and wash repeatedly with water. Suspend the magnesium hydroxide in 30 ml. isotonic sodium chloride.

(*b*) Buffered oxalated saline.

Take 5 ml. 1·85 % potassium oxalate; add 5 ml. glyoxaline buffer and make the volume to 250 ml. with isotonic sodium chloride.

(*c*) Buffered saline. Take 5 ml. glyoxaline buffer and dilute to 250 ml. with isotonic sodium chloride.

(*d*) Saturated ammonium sulphate solution.

(*e*) Oxalated plasma. In a clinical biochemical laboratory it is convenient to use mixed residues of human oxalated blood obtained in the routine work. The blood should not be more than a few days old. It is centrifuged and the plasma is separated.

Take 30 ml. plasma and add 6 ml. magnesium hydroxide suspension. Mix well and centrifuge off the magnesium hydroxide, which carries down a considerable proportion of the plasma prothrombin. Take 30 ml. of the supernatant plasma and gradually add 10 ml. saturated ammonium sulphate, mixing well. Fibrinogen is precipitated. Centrifuge, discard the supernatant fluid and dissolve the fibrinogen in 30 ml. buffered oxalated saline. When solution is complete, add 10 ml. saturated ammonium sulphate to precipitate the fibrinogen a second time. Centrifuge; discard the supernatant fluid, and dissolve the fibrinogen in 10 ml. buffered oxalated saline. Transfer the fibrinogen solution to a collodion sac and dialyse for at least an hour against about 400 ml. buffered oxalated saline. Discard the external fluid, replace it with buffered saline (without oxalate) and repeat the dialysis.

The fibrinogen preparation is now ready for use. It will keep one day at ordinary refrigerator temperature. It can be stored for about a week if sealed up in ampoules and placed in a vacuum flask with solid CO_2 on the refrigerator. If this method is adopted the preparation should be distributed in lots of about 2 ml. and each opened only as required.

The method of preparation of fibrinogen is essentially the same as that used by Warner *et al.*, but there are two differences in detail. They corrected the reaction of the plasma after adsorption with $Mg(OH)_2$ to *p*H 7·4. This is unnecessary because the addition of the $(NH_4)_2SO_4$ itself brings the reaction of the alkaline plasma back to the desired range. Secondly, the *p*H is controlled throughout the preparation by including glyoxaline buffer in the saline used for solution and dialysis. Under these conditions satisfactory preparations were regularly obtained. The dialysis was usually done in the refrigerator, but no difficulty arose from carrying out the other manipulations at room temperature.

The necessity for using a freshly made fibrinogen preparation is the only inconvenient feature of the method. In laboratories equipped with apparatus for the rapid desiccation of protein solutions (e.g. sera) it would be possible to prepare permanent preparations of desiccated fibrinogen [Ferguson & Erickson, 1939].

Procedure

Oxalated venous blood is collected as quickly as possible, with the minimum of oxalate necessary to prevent clotting. The plasma is separated. A series of dilutions of the plasma in isotonic sodium chloride is prepared, giving 1 vol. plasma in 25 vol., 1 : 50, 1 : 100 and 1 : 200.

From each of these, the following mixtures are made:

1 ml. diluted plasma.
1 ml. glyoxaline buffer.
2 ml. brain extract (containing Ca).

It will be noted that the dilutions of plasma at the stage of incubation are 1 : 100, 1 : 200, 1 : 400 and 1 : 800. These mixtures are incubated at 15–20° and at intervals samples are taken for clotting tests as follows. A sample of 0·2 ml. is measured into a test tube and placed in a water bath at 37°. Fibrinogen, 0·1 ml., is rapidly added from a pipette calibrated "to blow out". A stop watch is started at the moment when the fibrinogen is introduced and the time taken when the mixture gels. The end point is determined by dipping into the mixture a capillary pipette provided with a teat, drawing the fluid up slightly into the capillary, and lifting the tip of the pipette just out of the fluid. This manipulation is rapidly repeated until a solid gel adheres to the capillary tip. With a little practice the end point can be read very precisely.

In repeated clotting tests on the incubating mixtures it is found that the times become shorter as thrombin increases, then remain steady for some time and later lengthen. The result to be recorded is the clotting time for each mixture during the period of steady activity. It is always necessary to take repeated tests to ensure that the readings are made at the right stage, as the time required for development of maximal thrombin activity varies with different plasma samples. But in practice only a few tests are necessary on each incubating mixture, because the period of maximal activity is heralded by the appearance of a slight fibrin clot in the mixture. When this occurs, the tube is shaken to cause the little clot to retract and the clotting tests are begun. Each mixture is tested at intervals of 2 or 3 min. The period of maximal activity is reached earliest in the 1 : 100 dilution, and later with increasing plasma dilution. For this reason it is possible to start all four mixtures incubating at the same time, fitting in the clotting tests on all as they reach their maximal activity. For a

normal plasma the usual times required for the development of maximal activity are:

Dilution 1 : 100: 5–10 min.
1 : 200: 10–20 ,,
1 : 400: 20–30 ,,
1 : 800: 30–40 ,,

The standard clotting times in the clotting tests with fibrinogen during the period of maximal activity, for an average normal plasma, are:

Dilution 1 : 100: clotting time 26 sec.
1 : 200: ,, 42 ,,
1 : 400: ,, 67 ,,
1 : 800: ,, 107 ,,

The dilutions mentioned are those existing in the incubating mixture. The further dilution of 2 to 3 in the clotting test is not included in the recorded figures.

When the method is applied to a plasma of low prothrombin content, the clotting times are longer and the times required for the development of maximal activity are also longer. It is therefore important to note the appearance of the slight fibrin clot in the incubating mixture; this is especially useful as a guide to the time tests and should be made when dealing with a plasma of unknown prothrombin titre.[1]

Clotting times can be accurately read in the range 15–120 sec. For clotting times above 120 sec. it is difficult to obtain sharp end points. Therefore, when dealing with samples of plasma of low prothrombin titre, the higher dilutions cannot be used, but it will be possible to get readings with one or more of the dilutions mentioned in all cases.

Calculation

The titre of any plasma, expressed as a percentage of the normal average, is given by the formula $T/T' \times 100$, where T is the clotting time for the standard normal plasma at any given dilution, and T' the clotting time for the "unknown" plasma at the same dilution.

For example, a plasma from a case of obstructive jaundice gave the following readings:

Dilution	T' sec.	T sec.	$T/T' \times 100$
1 : 100	56	26	46
1 : 200	90	42	47
1 : 400	141	67	47½

In general, the values obtained from readings made at different dilutions agree well, though there is sometimes more variation than in the above example. The fact that the results are consistent for different dilutions shows that this method of calculation affords, at least, a practical means of comparing abnormal plasma with the standard normal. The question whether it also gives a truly quantitative scale of prothrombin titres requires an understanding of the different reactions involved. Three reactions need to be considered from the quantitative aspect, namely (1) the formation of fibrin from fibrinogen under the influence of thrombin, (2) the conversion of prothrombin into thrombin and (3) the effect of plasma antithrombin in high dilutions of plasma.

[1] The appearance of this slight fibrin clot, though generally a useful guide, should not be relied on absolutely. The clot is not noticeable in a 1 : 800 dilution of normal plasma, and may easily be misse n lower dilutions of a plasma poor in fibrinogen.

Factors involved in the estimation of prothrombin

(1) *The relation between thrombin concentration and the rate of clotting of fibrinogen.* For experiments on this reaction thrombin preparations were made as follows.

Oxalated plasma, 10 ml., is diluted to 100 ml. in cold distilled water; a drop of octyl alcohol is added, and CO_2 is passed through the mixture for about 5 min. to precipitate prothrombin. The precipitate is separated by centrifuging and dissolved in 10 ml. isotonic sodium chloride containing a little glyoxaline buffer; 0·5 ml. $M/10$ $CaCl_2$ is then added and the mixture warmed to 37°. The clot which forms is removed by rolling it out on a glass rod. The excess Ca is removed either by dialysis or by precipitation with an equivalent amount of oxalate.

Thrombin preparations made in this way are very stable, retaining their activity for several weeks on the refrigerator with only slight loss.

Table 1

Dilution of thrombin	Clotting time sec.
Undiluted	$7\frac{1}{2}$
1 : 2	$11\frac{1}{2}$
1 : 3	14
1 : 4	20
1 : 6	$25\frac{1}{2}$
1 : 10	37
1 : 15	55
1 : 20	$74\frac{1}{2}$
1 : 30	116

Experiments were made in which a series of dilutions of such preparations was tested by performing clotting tests with fibrinogen. The clotting tests were made with 0·2 ml. of the diluted thrombin and 0·1 ml. fibrinogen in the manner

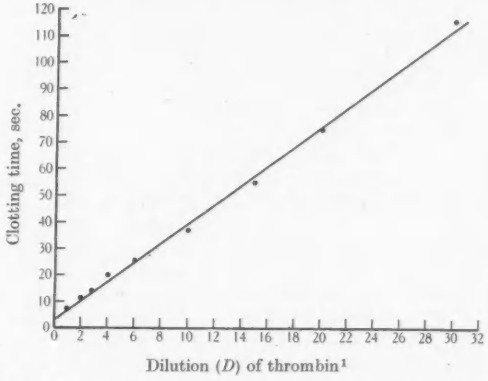

Fig. 1. Relation between dilution of a thrombin preparation and clotting time with added fibrinogen.

already described. A typical experiment is given in Table 1. The same result is shown graphically in Fig. 1, where the dilution of the thrombin (i.e. the volume to which one volume of the original preparation is diluted) is plotted against the clotting time obtained in tests with fibrinogen. The points in Fig. 1 lie very

[1] Here and subsequently the symbol D represents the volume to which 1 vol. of sample is diluted.

close to a straight line which cuts the time axis at 3 sec. As the shorter clotting times may easily be slightly overestimated, the true relation may well be a straight line passing through the origin—indeed, for clotting times above 25 sec. a straight line through the origin would fit the points equally well. That is to say, the dilution of the thrombin is directly proportional to the clotting time; or, the concentration of thrombin is inversely proportional to the clotting time. If the concentration of thrombin were plotted against the clotting time, the result would be a rectangular hyperbola. As it is obviously preferable to choose the method of plotting which gives the linear relation with thrombin, all the results to be described will be expressed in this way, as a relation between "dilution" (the volume to which one volume of a sample is diluted) and the clotting time in a test in which 0·2 ml. of the diluted sample is treated with 0·1 ml. fibrinogen.

These results with thrombin are in agreement with those obtained by Quick [1935] with similar preparations.

(2) *The conversion of prothrombin into thrombin.* Preparations of prothrombin were made as follows.

Oxalated plasma is first freed from fibrinogen by treatment with thrombin. A sample of 10 ml. plasma is treated with a few drops of thrombin (prepared as above) and the mixture warmed to 37°. When the clot has formed, the fibrin is removed by rolling it out on a glass rod. More thrombin is added, and any further fibrin clot removed in the same way. Repeated treatments with a drop of thrombin are continued until no further clot forms. The mixture is then kept for at least 15 min. to allow of complete destruction of the excess thrombin by the plasma antithrombin. The plasma is then diluted ten-fold with water, a drop of octyl alcohol is added and CO_2 is passed through the mixture for 5 min. Prothrombin is precipitated. The precipitate is separated by centrifuging and dissolved in 10 ml. buffered saline (using the glyoxaline buffer as already described).

From preparations made in this way, a series of dilutions was made in isotonic saline, and each mixture incubated with calcium and thromboplastin and tested at intervals for thrombin activity, exactly as in the method for estimating plasma prothrombin. It was found that the clotting times (with added fibrinogen) decreased rapidly during the early stages of incubation and then more gradually, until a maximal activity was reached. The time required to develop full activity was 30–90 min., and the thrombin then remained stable for at least an hour. It was also noticed that the time taken to reach maximal activity was the same for smaller as for larger amounts of prothrombin, and that the thrombin formed at any given stage of incubation was roughly proportional to the prothrombin present.

At maximal activity, the relation between the dilution of the preparation and the clotting time with fibrinogen was perfectly regular. Examples are given in Fig. 2, which show the results with four prothrombin preparations of different strength. For each preparation, the points fall on a straight line which cuts the time axis at some point between 0 and 4 sec. As with thrombin, the ratio D/T can be regarded as constant for clotting times above 25 sec. For any one prothrombin preparation, the prothrombin concentration (ϕ) in the incubating mixture is inversely proportional to the dilution (D). We know that the thrombin concentration (θ) is inversely proportional to the clotting time (T). For any one plasma the ratio D/T is constant, and this means that the ratio θ/ϕ is constant. This does not prove that the prothrombin is quantitatively converted into thrombin, but it does show that the thrombin formed is always proportional to the prothrombin present.

It is evident also that the value of the constant D/T for any preparation is proportional to the prothrombin titre of the preparation. The ratio of the titres of two preparations will be given either by the ratio T_s/T_u, where T_s and T_u are the clotting times for the "standard" and "unknown" respectively at the same dilution, or by the ratio D_u/D_s where D_s and D_u are the dilutions of the standard and unknown respectively which give the same clotting time.

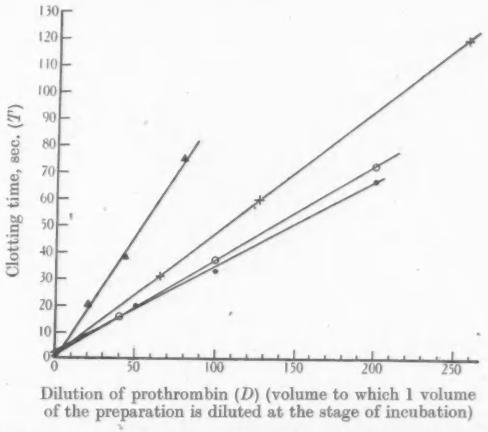

Fig. 2. Relation between the dilution of a prothrombin preparation and the clotting time with added fibrinogen during the period of maximal thrombin activity.

Now if, in diluted plasma, the prothrombin were completely converted into thrombin without loss, the prothrombin titre of an abnormal plasma could be calculated as a percentage of the standard normal equally well by fixing the dilution and comparing clotting times, or by fixing the clotting time and comparing the corresponding dilutions. It will be shown that for plasma the two methods do not give the same results because the ratio D/T (or θ/ϕ) is not constant for all dilutions of the same plasma, but decreases with decreasing dilution. Before considering more fully the results obtained with plasma, it will be convenient to describe experiments on the antithrombin activity of diluted plasma.

(3) *The antithrombin of plasma.* It is well known that plasma contains a substance capable of destroying thrombin. Under natural conditions of blood coagulation this "antithrombin" has the function of destroying the excess thrombin formed at the site of injury and so preventing the spread of coagulation to the general circulation. It does not act as an anticoagulant, probably because the velocity of the reaction between thrombin and antithrombin is less than that of the reaction between thrombin and fibrinogen. Quick [1938, 1] considers that the antithrombin of normal plasma is closely associated with the albumin fraction.

In undiluted plasma, and in any dilution less than 1 : 50, the rate of destruction of thrombin is rapid. If such plasma mixtures are incubated with calcium and thromboplastin, and the thrombin activity followed by performing clotting tests with added fibrinogen, an initial increase in thrombin is obtained, followed by a rapid fall, with no steady intermediate period of maximal activity.

At dilutions greater than 50, a steady period occurs, but it is evident that this may be due to temporary balance between the rates of formation and destruction of thrombin. It has already been mentioned that in the estimation of plasma prothrombin it is necessary to use the range of dilutions from 100 to 800, and that for samples low in prothrombin the lower dilutions of this range must be used. Experiments have therefore been made to determine the rate of destruction of thrombin by diluted plasma in this range.

Thrombin was prepared by the method already described, and the titre of the preparation standardized by clotting tests with added fibrinogen. Mixtures were made of diluted thrombin and diluted plasma, using the range of plasma dilutions from 1 : 100 to 1 : 800, and varying amounts of thrombin. These were incubated at 15–20° and at intervals samples were taken for clotting tests with fibrinogen. The thrombin present at any given stage, expressed as a percentage of that originally present, was calculated from the formula $T/T' \times 100$, where T is the clotting time in a control test for the thrombin without plasma, and T' the clotting time for the incubated mixture. The results are summarized in Table 2. Different samples of plasma varied somewhat in antithrombin activity,

Table 2. *Destruction of thrombin by diluted plasma*

Dilution of plasma	Percentage of the original thrombin remaining after incubation for			
	5 min.	10 min.	20 min.	30 min.
1 : 100	75–95	65– 80	60– 75	50–70
1 : 200	—	85–100	75– 90	70–85
1 : 400	—	95–100	90–100	85–90

and Table 2 shows the range of variation. Some destruction of thrombin occurred in dilutions from 1 : 100 to 1 : 400, and the effect diminished with increasing dilution. It is small at dilution 1 : 400. At dilution 1 : 800 there is probably still some slight effect. For each individual plasma the decrease in the effect with increasing dilution was observed.

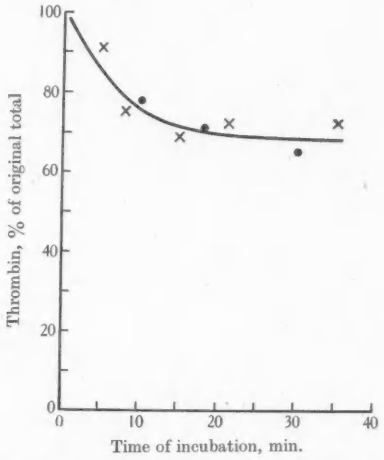

Fig. 3. Rate of destruction of added thrombin by plasma diluted 1 : 100. × Mixture 1. • Mixture 2. The original thrombin concentration in mixture 1 was twice that in mixture 2.

From experiments in which the dilution of the plasma was fixed and the amount of thrombin varied, it was found that the percentage of the original thrombin remaining at a given time was approximately the same whatever the original amount of thrombin. That is to say, when varying amounts of thrombin are incubated with the same amount of antithrombin, the thrombin lost in a given time is not a constant absolute amount, but a constant proportion of that originally present. This is illustrated in Fig. 3, which shows the loss of thrombin in two mixtures. Both contained the same plasma diluted to 100 vol., but the thrombin in mixture 1 was double that in mixture 2. The rate of loss, expressed as a percentage of the original value, is much the same for the two.

When one considers the times required for the development of maximal thrombin activity when diluted plasma is incubated with thromboplastin and calcium, it is evident that some antithrombin action may occur during this period, and although one cannot predict the quantitative effect in a system in which thrombin is being gradually formed, one would expect that the loss of thrombin would progressively increase with decreasing dilution of the plasma.

(4) *Thrombin formation from prothrombin in diluted plasma.* In the light of the preceding results, we can now consider the interpretation of the readings obtained in the estimation of plasma prothrombin. In Table 3 are given the figures on which the normal standards for the method are based. The samples of plasma were obtained from hospital patients, none of whom suffered from biliary obstruction or any other disease associated with a tendency to haemorrhage.

It is evident that here the ratio D/T is not constant but as the dilution decreases the clotting times become progressively longer than they would be if the ratio were constant. That is to say, the ratio θ/ϕ, which measures the thrombin formed relatively to prothrombin present, decreases as the concentration of antithrombin increases. The loss of thrombin is however quite regularly related to the plasma dilution, and it is possible to find an empirical formula expressing this relation for any given plasma.

Table 3. *Clotting times obtained at the period of maximum thrombin development with serial dilutions of normal plasma*

Case	Results of clotting tests on mixtures in which the volume to which 1 vol. of plasma was diluted at the stage of incubation was: 50	100 sec.	200 sec.	400 sec.	800 sec.	Mean prothrombin titre
1	—	22	35	61	90	116
2	—	28	45	66	104	97
3	—	33	44	78	123	87
4	—	25½	39	64	100	105
5	—	30	46	76	133	86
6	—	(22½)	36	60	88	116
7	—	25	38	60	110	105
8	—	28½	45	67	112	95
9	—	30	41	65	(106)	97
10	—	27	39	60	103	105
11	—	35	47	66	125	89
12	14	24	38	58	90	113
13	15	24	36½	55	93	115
14	20	31	47½	73	123	88
15	—	32	46⅓	77	132	85
Average normal	—	28	41½	66	109	(100)
Standard normal	16	26	42	67	107	—

Consider the series of dilutions (D) in decreasing order. They form a geometric progression with the factor $\frac{1}{2}$. It is found that the corresponding clotting times are in geometric progression with the factor 1/1·6. The "standard normal" clotting times at the foot of Table 3 form a series in which this relation applies strictly, and they fall close to the actual average normal results.

Table 4

Dilution (D)	Clotting time (T)	D/T	n
800	107	$\frac{800}{107}$	0
$400=\frac{800}{2}$	$\frac{107}{1\cdot6}=67$	$\frac{800}{107}\times0\cdot8$	1
$200=\frac{800}{2^2}$	$\frac{107}{1\cdot6^2}=42$	$\frac{800}{107}\times0\cdot8^2$	2
$100=\frac{800}{2^3}$	$\frac{107}{1\cdot6^3}=26$	$\frac{800}{107}\times0\cdot8^3$	3
$50=\frac{800}{2^4}$	$\frac{107}{1\cdot6^4}=16$	$\frac{800}{107}\times0\cdot8^4$	4

The corresponding values of D, T and the ratio D/T which accord with the empirical formula are set out in Table 4. Here the dilution 1 : 800, with its corresponding clotting time, 107, is taken as a starting point, but any other starting point would serve equally well. Whenever the plasma dilution is halved (antithrombin concentration doubled), the ratio D/T, which is proportional to θ/ϕ, is reduced to 0·8 of its value at the higher dilution. Doubling the antithrombin involves at each stage a loss of 20 % of the thrombin.

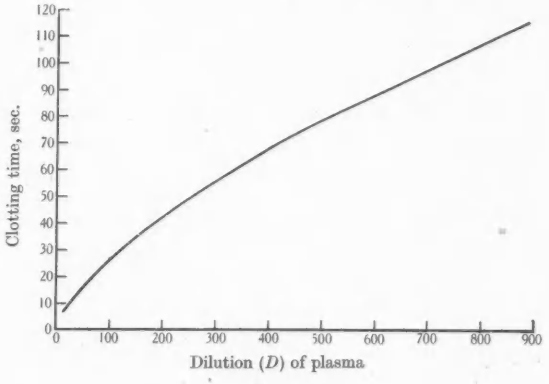

Fig. 4.

It is evident that if log D is plotted against log T, the resulting graph will be a straight line, and if D is plotted against T, the result is a logarithmic curve. It is possible to plot the full curve for all dilutions, and this has been done in Fig. 4.

The results for any individual sample of plasma agree closely with the empirical formula. For each, an "idealized" series of clotting times can be found which agrees with the observed series as well as could be expected in view

Table 5

	Dilution (D)				
	800	400	200	100	50
Case 12. Clotting time (T):					
Observed	90	58	38	24	14
Idealized	94	59	37	23	14
Case 13. Clotting time (T):					
Observed	93	55	36½	24	15
Idealized	94	59	37	23	14
Case 14. Clotting time (T):					
Observed	123	73	47½	31	20
Idealized	123	77	48	30	19

of the degree of accuracy of reading clotting times. The curve fitting was done by calculating from the readings at each dilution, according to the empirical formula, the clotting time which should correspond to dilution 1 : 800. The mean of these results was taken as the "idealized" clotting time at dilution 1 : 800, and this point was used to calculate the complete "idealized" series according to the empirical formula. In Table 5 the actual and idealized clotting times for three samples are set out. They were chosen because they included dilution 1 : 50 as well as the other four, and are quite typical.

Now in dealing with samples of plasma of low prothrombin titre, the same general relation applies. A geometric series of dilutions with the factor ½ corresponds to a geometric series of clotting times with the factor 1/1·6. Take any standard dilution D_s as the starting point, and let T_s and T_s' be the clotting times for the normal and abnormal samples of plasma respectively. Then at dilution $D_s/2$, the clotting times for the two samples will be $T_s/1{\cdot}6$ and $T_s'/1{\cdot}6$, and so on—the clotting times for the two vary always in the same proportion, and the ratio T/T' is constant for any dilution.

But the empirical formula is symmetrical in respect of D and T, and it can be shown that the ratio D'/D, where D and D' are the dilutions of the normal and abnormal which give the same clotting time, is constant for any clotting time. It gives a lower value than the ratio of clotting times at the same dilution.

The following example shows the results in a case of obstructive jaundice. At dilutions 1 : 400, 1 : 200 and 1 : 100 the clotting times were 104, 68 and 41 sec. respectively. The values of the ratio T/T' at each of the three dilutions are 0·64, 0·63 and 0·63. From the full standard normal curve in Fig. 4 it is found that the clotting times 104, 68 and 41 sec. are given by dilutions 1 : 770, 1 : 410 and 1 : 195 respectively, and the values of the ratio D'/D for the three clotting times as 0·52, 0·49 and 0·51. The discrepancy between the two ratios increases for lower prothrombin titres.

Now in view of the antithrombin effect it is highly improbable that the ratio D'/D is proportional to the prothrombin titre. Take an extreme example; a plasma giving a clotting time of 107 at dilution 100. The time of incubation required to reach maximal activity in such a case is about the same as for a normal plasma at dilution 1 : 800 giving the same activity. It is evident that there must be greater loss of thrombin at dilution 1 : 100 than at dilution 1 : 800.

What then of the ratio T/T' for samples of plasma compared at the same dilution? Obviously the fact that the ratio is constant for all dilutions does not prove that it is proportional to the prothrombin titre (as it is in the case of antithrombin-free preparations). It is quite possible that the antithrombin effect might be relatively greater throughout for the abnormal plasma, while still

conforming to the general relation which determines the form of the curve. If the formula $T/T' \times 100$ is taken as measuring the prothrombin titre as a percentage of the normal, the assumption is that the proportion of thrombin lost at a given dilution is the same for all prothrombin titres. If this is not so, the titres will be measured on a distorted scale.

An attempt has been made to determine whether the scale of titres is regular by experiments on the recovery of prothrombin added to plasma. This presents difficulties. If the method is adopted of adding a given quantity of a standardized preparation to a standardized plasma and determining the titre of the mixture, the difficulty arises of expressing the titre of the prothrombin preparation and that of the plasma in comparable units. Since the plotting of dilution against clotting time gives for prothrombin preparations a straight line, and for plasma a logarithmic curve, the titres of the two cannot be simply compared. The best approximation to a comparable standardization is to assume the absence of appreciable antithrombin activity in plasma at dilution 1 : 800, and to suppose that at this dilution the thrombin formed from plasma prothrombin is comparable with that formed from the separated prothrombin preparations. Such a calculation implies, not only that plasma antithrombin has negligible effect at dilution 1 : 800, but also that in the prothrombin preparations the conversion into thrombin is as complete as in plasma. The prothrombin preparations are very slowly converted, and since it has been shown by Mertz *et al.* [1939] that prothrombin is gradually inactivated by thrombin, it is quite possible that in these long incubations the thrombin formed early may prevent complete conversion of the remaining prothrombin.

Nevertheless, some experiments have been made with the addition of standardized prothrombin to plasma (Table 6). In these the titre of the prothrombin preparation has been expressed as a percentage of standard normal plasma on the assumption that the plasma and the preparation are comparable at dilution 1 : 800; and the titre of the plasma, with and without added prothrombin, has been calculated from the formula $T/T' \times 100$, where T and T' are the clotting times of standard and "unknown" at the same dilution of plasma. Some results show good agreement, and most are not grossly aberrant if the errors are considered in relation to the total prothrombin estimated. The general tendency is to high recoveries, and in two experiments (5 and 6) the recoveries are much too high. These discrepancies may possibly be accounted for by an underestimate of the titre of the prothrombin preparation. It is noteworthy that if the calculations had been based on the ratio of dilutions giving the same clotting time, instead of the ratio of clotting times at the same dilution, the discrepancies would have been very much greater.

In order to overcome the difficulty of expressing titres of plasma and separated prothrombin in comparable units, another method of approach has been used, with a view to discovering whether the method of calculating titres from the ratio of clotting times gives a regular, or a distorted scale. The question is whether the same increment of prothrombin has the same effect in different regions of the scale. In three experiments (nos. 5, 6 and 7 in Table 6) successive increments of prothrombin were added to a plasma of low titre. In exp. 8, the same increment of prothrombin was added to two samples of plasma, one of normal and the other of rather low titre.

This method of comparing increments is only capable of detecting fairly gross distortion of the scale, but with this reservation the results are extremely good. The range of prothrombin titres covered by the whole set of experiments is 16·5–137 % of the normal average, and for each experiment the two increments

Table 6. *Prothrombin titres expressed as percentage of standard normal plasma*

Exp.	Plasma alone	Plasma $+x$	Plasma $+2x$	Prothrombin added $x=$	Prothrombin recovered: 1st increment of x	Prothrombin recovered: 2nd increment of x
1	86	180	—	(80)	94	—
2	70	167	—	(76)	97	—
3	42	87	—	(46)	45	—
4	32	91	—	(51)	59	—
5	$16\frac{1}{2}$	$40\frac{1}{2}$	$63\frac{1}{2}$	$(13\frac{1}{2})$	24	23
6	45	93	137	$(32\frac{1}{2})$	48	44
7	48	79	116	$(37\frac{1}{2})$	31	37
8 (*a*)	63	85	—	(18)	22	—
(*b*)	85	111	—	(18)	26	—

agree reasonably well. In view of the fact that the determination of the increment is subject to the risk of summation of errors on two estimations, the results are suprisingly regular. It seems therefore that the method gives not only a practical index for comparing normal with abnormal plasma, but also a reasonably accurate quantitative estimation.

Other methods of prothrombin estimation

In the method of Warner *et al.*, and in the modification of it described in the present paper, the two essential stages in coagulation, namely, the formation of thrombin and the coagulation of fibrinogen by thrombin, are separated. Other methods have been based on the coagulation of plasma in the presence of thromboplastin and calcium under controlled conditions. In such coagulation systems the two reactions are proceeding in the same mixture, and during the short time required for clotting only part of the plasma prothrombin is converted into thrombin. What is measured is essentially the rate of thrombin formation.

One group of methods [Schønheyder, 1936; 1938; Dam & Glavind, 1938] depends on working with a concentration of tissue extract below the optimum, and determining the concentration of a standardized extract required to produce a standard clotting time with the plasma. The method requires precise standardization of the extract against normal plasma, and also requires that the plasma should be obtained free of thromboplastin.

Another group of methods is derived from that of Quick [Quick *et al.* 1935; Quick, 1938, 2]. The principle of the method as originally described was that when oxalated plasma was treated with calcium salts and an excess of thromboplastin, the only variable affecting the coagulation time was the prothrombin titre of the plasma. The relation between clotting times and prothrombin titres was worked out on the basis of results with serial dilutions of normal plasma. This calibration curve was not a convenient one to work with, as large differences in prothrombin concentration produced relatively small differences in clotting time. On Quick's scale, a prothrombin titre of 100 % of normal gives a clotting time of 12 sec.; 50 % of normal gives 15 sec. Moreover, various subsequent reports from other authors have shown that it is necessary to standardize the tissue extract; thromboplastin is required not in simple excess but in optimal concentration. The writer has been unable to reproduce Quick's clotting times even with optimal amounts of tissue extracts; the times found for undiluted plasma varied from 22 to 40 sec., and this agrees with the findings of Illingworth [1939]. Moreover, it was found that variations in the tissue extract affected not

only the clotting time for undiluted plasma, but the relation between clotting time and plasma dilution in serial dilutions of plasma. In fact it is very difficult to apply the method in a strictly quantitative fashion, though it gives a convenient means of detecting gross deficiencies of prothrombin.

Methods both of Schønheyder's type and Quick's type require standardization of the thromboplastin against normal plasma. Such a standardization makes no allowance for the range of normal variation, and it is impracticable to cover the full normal range whenever a standardization is required.

Another objection to any method which depends on the rate of thrombin formation is that other factors, beside the concentrations of prothrombin and thromboplastin, may affect the results. The differences in the rate of conversion of prothrombin into thrombin in different species have already been mentioned, and there is evidence that in human plasma under different conditions there may be variations in the rate of prothrombin conversion [Ziffren *et al.* 1939; Smith *et al.* 1939; Owen *et al.* 1939]. These authors recommend a modification of Quick's method as a useful practical measure of the tendency to haemorrhage, but do not regard this type of method as giving a true measure of prothrombin titres.

It is evident therefore that a method of the "two-stage" type is to be preferred, and it is hoped that the technique here described will meet the requirements of workers who need greater accuracy than can be obtained in methods of Quick's type.

Summary

1. The estimation of plasma prothrombin, by a modification of the method of Warner *et al.*, is described.

2. The quantitative aspects of the different reactions involved are considered.

3. Evidence is presented that the method gives a quantitative measure of prothrombin titres, or at least that there is no serious departure from a genuinely quantitative scale.

4. The principles underlying other methods are briefly considered.

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187. THE BIOLOGICAL ACTIVITY OF THE OXIDATION PRODUCTS OF *α*-TOCOPHEROL

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(*Received 31 October 1940*)

THE appearance of the results reported by Golumbic & Mattill [1940] has clarified to some extent the position regarding the biological activity of the oxidation products of α-tocopherol. The major discrepancy concerned the yellow *p*-tocoquinone which Karrer *et al.* [1938], John *et al.* [1939], Wright & Drummond [1940], Karrer & Geiger [1940] and Golumbic [1940] had reported as being unable to replace vitamin E in rat experiments, whereas, in strong contrast, Emerson *et al.* [1939] and Evans *et al.* [1939] maintained that its activity was little less than that of α-tocopherol.

Golumbic & Mattill [1940] now suggest that the oxidation resulting from the reagent employed by Emerson *et al.* [1939], namely, $FeCl_3$ acting in methyl alcohol at room temperature, was incomplete and that the activity of their preparation in biological tests was due to residual α-tocopherol. However, this explanation fails to account for the approximately equal biological activities shown by their preparation of the quinone and the parent substance.

The spectroscopic data given for the sample of the α-tocoquinone examined by Emerson *et al.* [1939] clearly show that if there was unchanged α-tocopherol present the amount was probably less than 10 or 15%.

We have repeated this oxidation, following as closely as possible the directions given by Emerson and his colleagues. 250 mg. were allowed to react with 1·25 g. $FeCl_3$ in 12·5 ml. methyl alcohol for 90 min. at room temperature. The quinone, separated by diluting the reaction mixture with water and extracting with ether, was a yellow oil showing on spectroscopic examination selective absorption at 262 and 268 mμ. At both wave-lengths the extinction coefficient was the same, $E^{1\%}_{1\,cm.}=375$. The value deduced from the data given in the paper by Emerson *et al.* [1939] is $E^{1\%}_{1\,cm.}=310$.

Our quinone preparation was inactive in doses of 5 mg. when tested on rats (see B, Table 1). We are satisfied, therefore, that this oxidation product does not possess a biological activity of the same order as the parent alcohol. Moreover, our spectroscopic data support the view of Golumbic & Mattill [1940] that the preparation examined by Emerson *et al.* [1939] was incompletely oxidized. It still remains difficult to account for the equal activities recorded by these workers.

Oxidation with a warm methyl alcoholic solution of $AgNO_3$, as employed by Cuthbertson *et al.* [1940], gives the same quinone. The product is spectroscopically identical with that obtained by cold $FeCl_3$ and it is also biologically inactive (see C, Table 1).

More vigorous oxidation with $AgNO_3$, or with HNO_3, yields a red compound which appears to have the structure of an *o*-quinone [Smith *et al.* 1939].

Table 1

Dose	Oxidizing agent	Rat no.	Uterine index
A	—	3	(9·49 + 10)/5 = 3·90
		7	(8·42 + 9)/5 = 3·48
		34	(6·35 + 6)/5 = 2·47
		16	(9·10 + 8)/5 = 3·42
		39	(6·32 + 7)/5 = 2·66
			Mean = 3·19
B	$FeCl_3$	9	(1·00 + 0)/5 = 0·20
		23	(0·90 + 0)/5 = 0·18
		37	(1·14 + 0)/5 = 0·23
		26	(5·76 + 8)/5 = 2·75
		28	(4·07 + 0)/5 = 0·81
		14	(1·69 + 0)/5 = 0·34
			Mean = 0·75
C	$AgNO_3$	4	(1·30 + 0)/5 = 0·26
		19	(0·89 + 0)/5 = 0·18
		20	(2·65 + 0)/5 = 0·53
		35	(1·30 + 0)/5 = 0·26
		12	(0·67 + 1)/5 = 0·33
		29	(1·86 + 0)/5 = 0·37
		30	(4·48 + 0)/5 = 0·90
		45	(6·75 + 8)/5 = 2·95
			Mean = 0·72
D	HNO_3	49	(1·47 + 0)/5 = 0·29
		6	(2·73 + 3)/5 = 1·15
		17	(6·02 + 8)/5 = 2·80
		25	(7·09 + 8)/5 = 3·02
		36	(1·04 + 0)/5 = 0·21
		13	(2·33 + 0)/5 = 0·47
			Mean = 1·32

We have prepared this compound. It showed no selective absorption in the ultraviolet, but there was a band with poor persistence at about 405 mμ. Doses of 5 mg. gave a positive response in rat tests (see D, Table 1), but the activity was less than that of the parent compound.

The biological tests were carried out according to the technique devised by Mason [1939] who defines the Uterine Index by the formula:

$$\tfrac{1}{5}\,(\text{Wt. of uterine contents} + \text{No. of viable foetuses (in g.)}).$$

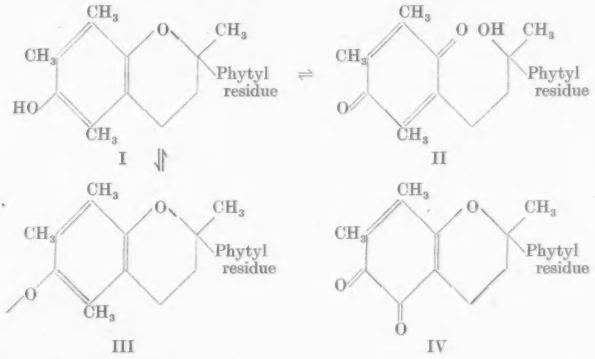
Values less than 0·35 indicate "completely negative" responses; those greater than 0·35 but less than 1·0 indicate "subminimal", and those greater than 1·0 "positive" responses. α-Tocopherol acetate and the *o*-quinone give positive responses, but the $FeCl_3$ and $AgNO_3$ oxidation products show subminimal activity.

In view of the activity of the *o*-quinone, attempts were made to reduce this compound to the corresponding quinol. So far they have been unsuccessful, in that the reduced compound has not been isolated in a stable form. By reduction with H_2 in the presence of Raney nickel, a pale yellow product was obtained showing a broad band at 262 mμ, but its unstable character has so far been an obstacle to satisfactory isolation.[1]

Our investigation of these oxidation products had as its main object the further examination of the hypothesis that the tocopherols and certain of their derivatives act as oxidation-reduction systems in the tissues [Drummond, 1939].

[1] Unfortunately this work has been temporarily suspended owing to war conditions.

The inactivity of the *p*-quinone, now amply confirmed, seems to dispose of the idea that the quinone-quinol, I ⇌ II change occurs in the tissues. This has led Golumbic & Mattill [1940] to postulate that the free phenoxyl radical, III, may be the biologically important unit of a reversible system.



The fairly high biological activity of the product of oxidation by HNO_3, which the work of Smith *et al.* [1939] suggests may have the constitution IV, is

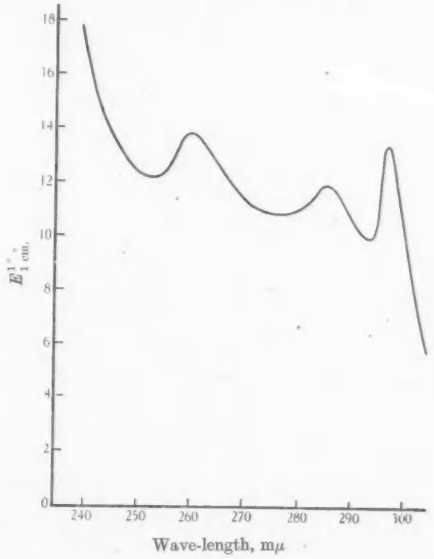


Fig. 1.

doubtless related to the preservation of certain features of the structure of tocopherol, i.e. the integrity of the oxygen ring and the phytyl side-chain.

In view of the fact that storage of the tocopherols can take place in the body fat and certain other tissues of rats after the administration of relatively large doses, it was thought to be of interest to see whether this active quinone would also be retained.

A group of 4 adult female rats, all of which had been deprived of vitamin E for at least 10 months, were given orally a dose of 10 mg. of the *o*-quinone on each of 5 successive days. For purposes of comparison, a similar group of rats was employed for experiments in which (*a*) α-tocopherol, (*b*) the *p*-quinone prepared by oxidation with $FeCl_3$ and (*c*) the *p*-quinone prepared by $AgNO_3$ oxidation were administered.

The body fats and liver fats of these animals were examined by the method described by Cuthbertson *et al.* [1940]. The results with the liver fats were unsatisfactory owing to interference by the large amounts of vitamin A present. None of the body fats showed any evidence of selective absorption on spectroscopic examination, but that from the group to which α-tocopherol acetate had been given yielded unsaponifiable fractions which darkened appreciably on oxidizing with $AgNO_3$.

Another experiment of a similar nature was made in which a single dose of 10 mg. of each of the four compounds was given. A week later, the body fats were removed and the unsaponifiable fractions were examined. The only positive result was in the case of those obtained from the animals which had had 10 mg. each of the *o*-quinone. The unsaponifiable fraction showed the triple band illustrated in Fig. 1. Oxidation with $AgNO_3$, as practised in examining material for tocopherol, did not alter the character of the absorption.

Summary

1. The products formed by the oxidation of α-tocopherol with $AgNO_3$ and $FeCl_3$ are spectroscopically identical. Both show subminimal activity in rat tests when given in oral doses of 5 mg. We cannot confirm the finding of Emerson *et al.* [1939] that α-tocoquinone prepared in this manner has substantially the same activity as the parent compound.

2. It does not appear that any of the known oxidation products of α-tocopherol are likely to take part in a reversible oxidation system in the body. The biological activity of the red *o*-quinone obtained by oxidizing α-tocopherol with nitric acid is relatively high, but this is probably attributable to the integrity of the oxygen ring and the phytyl side-chain. The only plausible suggestion regarding the nature of reversible systems affecting the oxidation of vitamin E is that of Golumbic & Mattill [1940] which relates α-tocopherol to the corresponding free phenoxyl radical.

To the Medical Research Council we are indebted for a grant from which part of the expense of this investigation was defrayed. We are also very grateful to Roche Products Ltd. for a generous supply of pure α-tocopherol acetate.

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188. THE EFFECT OF RICKETS AND OF ERGOSTEROL ON THE BRAIN AND LIVER CALCIUM OF YOUNG RATS

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(*Received 31 October 1940*)

In spite of the production of Ca deposits in the soft tissues by means of parathyroid extracts and irradiated ergosterol, the quantitative determination of Ca in the body tissues has not been the subject of many investigations. The concentration of Ca in normal tissues varies in a way which was not to be expected from the narrow range within which it fluctuates in the serum. On the other hand the changes in tissue Ca concentration produced by experiment and by disease are small, and the variability of its concentration in healthy tissues has made the significance of small changes difficult to assess except by statistical means.

The present contribution contains the results of Ca analyses of the brain and liver tissue of rats with low-phosphorus rickets and acute ergosterol poisoning.

Experimental

The rats were of Wistar strain and were killed when 9–12 weeks old and weighing 80–120 g. Normal controls were fed on dog biscuit, dried milk powder, poultry meal and fresh carrots. Cod liver oil was given to them twice a week and they were housed in a sunny room.

Rickets. Groups of rats received the high Ca low P diet RR 401 [Jephcott & Bacharach, 1926] from the age of 4–6 weeks. They were shaded from light. When killed 28–56 days later the presence of advanced active rickets was shown by the very low level of the serum inorganic P and by the presence of marked rachitic changes in the epiphyses.

Ergosterol. These animals were placed in separate cages at the age of 9 weeks. On the first evening of the experiment all food was removed from the cage except a small amount of ground dog biscuit containing 0·5 ml. of 10,000 *X* irradiated ergosterol solution. This was completely consumed by the next morning when poultry meal and carrot were offered and as a rule consumed in the course of the day. On the second evening surplus food was removed and a second dose of ergosterol on biscuit was given. By the second morning only part of the ergosterol had been taken and the rats were ill and refused other food. They passed small amounts of blood by the bowel. By the third morning all were very ill and some had died. The survivors were killed and these only were taken for the analyses.

Ergosterol controls. The ergosterol animals lost a large amount of weight during the experiment, part of this loss being due to refusal of food. To control this factor some similar animals were caged separately and allowed only the food corresponding to that actually eaten by the poisoned animals. These controls failed to gain weight or lost a few grams, but the weight loss was not as great as in most of the ergosterol animals.

The animals were killed by cutting the throat and blood was collected for Ca and inorganic P determinations. The brains were removed with care to avoid contamination with bone. Three brains were mashed together and the pooled material was passed through fine washed gauze. Sufficient material was obtained for duplicate ashing and analysis which were carried out as previously described [Linder, 1935]. The livers were treated in the same way.

McCance & Shipp [1933] state that if tissues to be analysed for Ca are not reduced to a white ash much Ca is lost by inclusion in, or adsorption on, particles of carbon. The ash obtained by the method used here contained a small amount of carbon. Duplicate determinations were made by this method and by the Stolte [1911] method, using ammonium nitrate to help the process of ashing and continuing until all the carbon was destroyed; the results were the same. The routine method was also compared with a modification in which heating was continued for 2 hr. in the furnace at a dull red heat; the carbon was destroyed but no more Ca was recovered. A satisfactory recovery of added Ca was also obtained.

Small portions of the ergosterol livers were taken for histological examination.

Results

The results are given in Tables 1 and 2 and in Fig. 1. The tables show the number of determinations in each group and the mean Ca concentration in the fresh tissue with its standard deviation. The mean water content is given. The results in the different groups were compared by the statistical method described by Tippett [1931] for the comparison of the means of small samples. The value of t was calculated and the corresponding value of p was taken from Fisher's [1938] table. When p is less than 0·05 the means may be taken as significantly different. The results are grouped in pairs for comparison, and for each pair the right-hand columns of the tables give the difference between the means of the pair and the values of t and p derived from them. It will be seen that p in the cases in which differences are present is 0·01 or less, indicating a much greater degree of statistical probability.

Table 1. *Means of the series of brain Ca determinations and their statistical comparison*

Series	No. in series	Mean water content %	Mean Ca mg./100 g.	σ	Difference of mean Ca from normal	t	p
Normal	21	78·6	4·71	0·43	—	—	—
Rickets	12	78·8	5·30	0·62	0·59	3·22	0·01
Ergosterol	13	78·7	6·42	0·81	1·71	8·44	<0·01
Ergosterol control	4	78·7	4·63	0·45	0·08	0·337	0·7

Table 2. *Means of series of liver Ca determinations and their statistical comparison*

Series	No. in series	Mean water content %	Mean Ca mg./100 g.	σ	Difference of mean Ca from normal	t	p
Normal	14	71·7	3·28	0·51	—	—	—
Rickets	27	72·3	3·36	0·90	0·08	0·307	0·7
Ergosterol	24	72·7	4·97	0·95	1·69	6·06	<0·01
Ergosterol control	13	71·9	4·16	0·72	0·88	3·69	<0·01
Ergosterol control	—	—	—	—	0·81	2·67	0·01

Rickets. The mean serum Ca of the rachitic rats was 9·9 mg./100 ml.; this was practically the same as the normal mean, 9·7 mg. The mean inorganic P of the rachitic sera was 1·04 mg. and that of the normal sera was 5·5 mg.; the presence of rickets was therefore evident. Table 1 shows that the mean brain Ca in this type of rickets was greater than that of the normal brains by 0·59 mg./100 g.; p is less than 0·01, therefore this increase, although small, is unlikely to be due to chance. The mean water contents of the two groups were the same. From Table 2 it can be seen that the mean Ca content of the liver was unchanged in rickets, and there was no significant change in the water content.

Ergosterol. In the ergosterol group the mean serum Ca concentration was 13·2 mg./100 ml. This was 3·5 mg. higher than the mean of the normal group

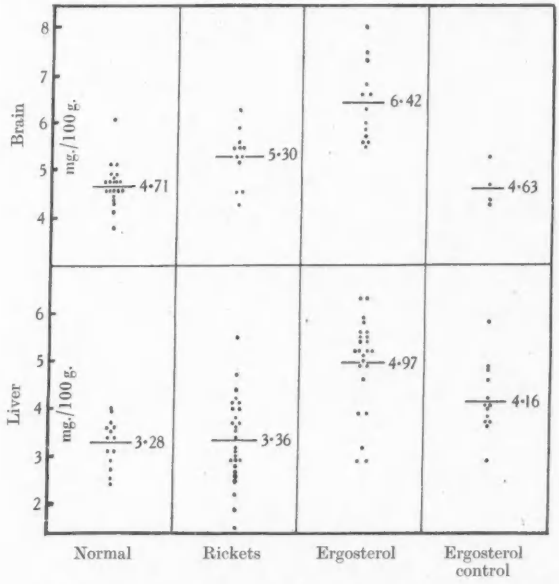

Fig. 1. Individual observations on the Ca contents of the brain and liver of young rats. Effect of rickets and of ergosterol.

and is an indication of the degree of ergosterol toxaemia which had been established. The mean brain Ca was 1·71 mg. higher than the normal; the value of t shows that this difference is highly significant. That this was not due to partial starvation is shown by the last comparison in Table 1, which demonstrates that the mean brain Ca of the partially starved controls did not differ appreciably from that of the normal animals.

The concentration of Ca in the livers was also increased, the mean increase being the same as in the case of the brains. In the case of the liver, however, the mean Ca concentration of the partially starved controls showed a significant increase over that of the normal group, though the difference between the means of the ergosterol and partially starved groups was still sufficiently large to remain of significance. Of the increase in liver Ca it seems that half was directly due to

the ergosterol and that half may have been due to the lowered intake of food, the result of the ergosterol.

The water content of the tissues is included in the tables. The results have been examined on the basis of dry tissue, but the validity of the comparisons was not affected.

Microscopic examination of the livers showed diffuse areas in which the cytoplasm of all the cells and the nuclei of some of them stained very poorly; in two livers small areas of calcification were seen, and in a third there was a comparatively large area in which the cells were necrotic and there was extensive deposition of Ca.

Discussion

In the following remarks the Ca figures quoted from the literature have been reduced to mg./100 g. of fresh tissue. When the authors gave figures for dried tissue the values for fresh tissue were calculated by dividing the figures by 4.

Hess *et al.* [1932] made an extensive study of the Ca concentration in rat brain. The brains of their normal rats contained 25–65 mg./100 g. When rickets was produced by high Ca low P diets the brain Ca was reduced to between 9 and 19 mg., and a similar reduction followed a low Ca high P diet. An abrupt change from a high Ca low P diet to a normal diet caused tetany, the serum Ca falling and the brain Ca remaining low. They found that Ca could be rapidly removed from the brain by rachitogenic diets, but could only be restored slowly by normal diets and vitamin D.

Beumer [1936] found that the mean Ca content of 27 normal rat brains was 3·55 mg. and that of 27 rachitic rat brains was 2·08 mg. He agreed with Hess in regard to the lowered concentration in rickets, but the amounts of Ca which he found were very much smaller. Farago [1936] reported that the different parts of rat brain varied considerably in their Ca contents; the cortex contained 6 mg. and the cerebellum 0·4 mg. He does not give figures for the whole organ but they must have been about the same as those of Beumer [1936] and those reported here. Watchorn & McCance [1937] gave the brain Ca of black and white piebald rats as 56 mg. and Patwardhan & Chitre [1938] reported 13–20 mg.

The following amounts have been found in the brains of other animals: cat 8–17 mg. [Heubner & Rona, 1923] and 4–7 mg. [Katzenelbogen, 1932]; dog 2–8 mg. [Underhill & Jaleski, 1933], 14 mg. [Reed *et al.* 1933] and 26 mg. [Hetenyi, 1935]; rabbit 5·9 mg. [Denis & Corley, 1925]; guinea-pig 4–11 mg. [Linder, 1935]; calf 16 mg. and sheep 10·8 mg. [McCance & Shipp, 1933]; man 10·6 mg. [Magnus-Levy, 1910] and 4–6 mg. [Eaves, 1931].

Hess *et al.* [1932] pointed out that there was no reason to anticipate a fall in tissue Ca on a high Ca low P diet, and the results obtained in the present experiments do not show such a decrease either in brain or liver. On the contrary the brain Ca was slightly increased.

Investigations into the Ca content of rat liver tissue are even fewer. Favarger & Rutishauser [1937] found 4·5 mg. in normal rat liver and Patwardhan & Chitre [1938] 7 mg. The mean of the present determinations was 3·3 mg. and no change was found in low P rickets.

In other animals the following results for liver Ca have been recorded: dog 8·6 mg. [Voit, 1880], 22 mg. [Hetenyi, 1935] and 4·5 mg. [Reed *et al.* 1933]; rabbit 7·5 mg. [Denis & Corley, 1925] and 5 mg. [Smith & Elvove, 1929]; guinea-pig 4·2 mg. [Linder, 1935]; ox 8·1 mg. [Corley & Denis, 1925] and 8·7 mg.

[McCance & Shipp, 1933]; and man 7·1 mg. [Stoeltzner, 1899] and 7·2 mg. [Magnus-Levy, 1910].

The effect of large doses of ergosterol on the tissues has been extensively studied by histological methods [cf. Reed *et al.* 1933]. The extent of the calcification depends to a large extent on the nature of the diet, for the most extensive calcification has been found when the P intake was high and this occurred even if the intake of Ca was minimal [Shelling, 1930]; on a low P diet even with excess of Ca there may be no deposits [Smith & Elvove, 1929]. Similarly the height of the serum Ca was no indicator of the amount of tissue deposit. Harris & Innes [1930] added increasing amounts of Ca phosphate to a low Ca high P basal ration and found that ergosterol calcification increased with the Ca intake, but as phosphate was added as well as Ca these results are not in complete disagreement with those of Shelling [1930] and of Smith & Elvove [1929]. It seems likely that greater calcification occurs in prolonged, moderately severe overdosage, although deposits appear very quickly when large and rapidly fatal doses are given.

Localized histological deposits of Ca may not involve a large increase of Ca in the whole organ, but few analyses of tissues after dosing with ergosterol have been made. Reed *et al.* [1933] report a mean increase from a normal of 14 mg. to 31 mg. in the brains and from 4·5 to 30 mg. in the livers of dogs which were treated with large doses of ergosterol for periods varying from 8 to 73 days; the increases in the aorta, brain, muscle and spleen were statistically the most significant, but the largest increase was in the kidney. Smith & Elvove [1929] found 5 mg. in the livers of normal rabbits and 5–14 mg. after repeated excessive doses of ergosterol; the largest increases were found in the aorta, kidney and lungs. Hetenyi [1935] found 26·4 mg. in the brains and 22·4 mg. in the livers of normal dogs, and 27 and 31 mg. respectively after ergosterol; these increases he did not accept as significant. There were gross increases in the lungs, kidneys and muscles.

In the present series ergosterol was given in such doses that it appeared that all the animals would have died within a week, and many actually died before the end of the experiment. These were not included in the data because the organs would have contained much more blood than those of the rats killed by bleeding. The brains showed a definite increase in Ca which appears to have been directly due to the ergosterol and not to reduction of food consumed or to change in the water content of the tissue. The livers showed a definite increase but part of this was the result of reduced food consumption since normal rats with their diet restricted to that taken by the ergosterol-poisoned animals showed a significant increase in their liver Ca. However, after allowing for this there still remained a significant increase directly due to the ergosterol.

Although the absolute increase of Ca was not large, being 1·71 mg. in the brains and 1·69 mg. in the livers, the percentage increases, 36 for the brains and 51 for the livers, were quite considerable. They are, however, much smaller than those reported for prolonged experiments. Reed *et al.* [1933] found an increase of 122 % in the brain and 570 % in the liver after prolonged treatment and Smith & Elvove [1929] found increases up to 200 % in the liver after repeated excessive doses. Hetenyi [1935], the severity of whose treatment must have been comparable with that in the present experiments, found increases of 2 % in the brain and 38 % in the liver; these results are similar in magnitude to those reported here.

SUMMARY

1. The mean concentration of Ca in the brains of normal white rats was found to be 4·7 mg./100 g. of fresh tissue. In fresh liver it was 3·3 mg.

2. The brain Ca was decreased and the liver Ca unchanged in rickets produced by a high Ca low P diet.

3. The brain Ca was increased in acute ergosterol poisoning. The liver Ca was also increased, but part of the increase was the result of diminished consumption of food. The changes were smaller than those reported in less acute experiments.

I wish to express my thanks to Dr A. L. Bacharach for his advice in regard to the care of the rats and the construction of the rachitogenic diet; to Messrs Mead Johnson and Company for the gift of 10,000 *X* ergosterol; and to Mr E. J. Duncan for his technical assistance.

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189. PROLIFERATION-PROMOTING PROPERTIES AND ULTRAVIOLET ABSORPTION SPECTRA OF FRACTIONS FROM YEAST

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(*Received 26 October 1940*)

Work in these laboratories on proliferation-promoting factors has been concerned largely with a study of substances which appear to be produced by cells as a result of injury (intercellular wound hormones). It has also been necessary to examine factors of the bios type from "uninjured" cells. In many cases the different preparations have also been tested for their effects on the respiration of yeast and tissues and, in some instances, for their effects on glycolysis and fermentation. In most of the reported findings these various stimulating properties have been found to accumulate in different fractions.

Spectroscopic study of crude proliferation-promoting substances from ultraviolet-injured yeast cells has shown that the increase in biological potency parallels the increase in ultraviolet absorption at about 2600 Å. and this is substantiated by chemical tests [Cook *et al.* 1939; Loofbourow, Cook & Stimson, 1938]. Indications are similar with proliferation-promoting substances produced by mechanical injury [Loofbourow, Cook, Dwyer & Hart, 1939]. These facts have suggested that the growth factors from injured cells owe their activity to nucleic acid-like substances although not to nucleic acids themselves since nucleic acids and related substances possess only a very low order of activity under the conditions of assay [Loofbourow *et al.* 1940]. Similar conclusions are drawn with respect to proliferation-promoting substances prepared by the irradiation of animal tissues [Cook *et al.* 1939; Loofbourow *et al.* 1937; Loofbourow, Cueto & Lane, 1939; Loofbourow *et al.* 1940]. In this connexion reference should be made to the findings of Fischer [1939] and of Caspersson [1936; 1939] relating nucleic acids or nucleoproteins to proliferation.

Preliminary experiments showed that a bios preparation from yeast had marked absorption at 2600 Å. while bios prepared from sugar showed only end absorption [Loofbourow *et al.* 1937] as have some other bios preparations (unpublished). On the other hand, a bios sample from malt combings showed peaks at 2800 and 3000 Å. which might be due in part to cyclic amino-acids [Loofbourow *et al.* 1937]. It is of significance that Miller [1936] has found β-alanine and possibly *l*-leucine to act as components of the bios complex (Bios IIa). Williams & Rohrmann [1936] earlier pointed out the importance of β-alanine for yeast growth, and β-alanine has been recognized as a component of pantothenic acid [Weinstock *et al.* 1939] which has now been synthesized [Williams & Major, 1940; Williams, Eakin & Snell, 1940; Williams, Mitchell, Weinstock & Snell, 1940; Stiller *et al.* 1940]. The simple acyclic amino-acids show essentially end absorption in the ultraviolet [Ellinger, 1937] and pantothenic acid, as would be expected, appears to show little characteristic absorption

[Williams *et al.* 1939]. Other pure compounds having bios activity are inositol (Bios I) [Eastcott, 1928], aneurin (Bios V) [Miller, 1937; Schultz *et al.* 1938], and vitamin B_6 [Schultz *et al.* 1939; Eakin & Williams, 1939]. Inositol shows only end absorption (unpublished data). Aneurin is characterized by maxima at 2350 and 2670Å. [Wintersteiner *et al.* 1935]. The spectra of vitamin B_6 are more complex and alter with *p*H. At *p*H 2·1 there is a single peak at 2920 Å.; at *p*H 4 there are peaks at 2920 and 3260 Å.; at *p*H 5·1 there are maxima at 2550, 2920 and 3260 Å.; at *p*H 6·75 the middle peak disappears, leaving those at 2550 and 3260 Å.; at *p*H 10·2 the two peaks are shifted to 2460 and 3110 Å. [Stiller *et al.* 1939; Kuhn & Wendt, 1939].

None of the pure compounds identified as parts of the bios complex absorbs appreciably at 2600 Å. and none of them which has been tested by us (*β*-alanine, *l*-leucine, inositol and aneurin) shows proliferation activity on yeast under the conditions to be described [Loofbourow *et al.* 1940]. Until recently no evidence had been presented definitely to connect the biological activity of bios preparations with absorption at this wave-length, thus contrasting with the proliferation-promoting factors obtained from injured cells. Recently, however, Rainbow & Bishop [1939] carried out fractionations which gave highly purified Bios IIA and Bios IIB. The former, probably identical with pantothenic acid, exhibited only end absorption, whereas the latter, possibly equivalent to the biotin of Kögl & Tönnis [1936], showed an absorption maximum at 2620 to 2650 Å. Since many of the manifestly crude proliferation-promoting preparations obtained in our laboratories from injured cells show considerably greater extinction at 2600 Å. than Rainbow's highly purified Bios IIB, it is not yet clear to us whether the absorption of Rainbow's preparation is characteristic of his fraction or may be caused by highly absorbing impurities. Indeed, the extinctions of some of the fractions reported in the present paper equal or exceed that reported by Rainbow. Williams, Eakin & Snell [1940] have pointed out that substances other than the known bios components are important for rapid yeast growth.

Thus far it has been impossible to correlate respiration-stimulating activity of yeast fractions, either on tissues or yeast, with absorption at 2600 Å. [Cook & Walter, 1939] and this is supported by the data of the present paper; biological tests with nucleic acids and related substances have likewise been negative [Cook *et al.* 1940].

In a study of respiration [Cook *et al.* 1938], five fractions were obtained from Fleischmann's baker's yeast by a simplification of the procedure applied by Lucas [1924; also Miller *et al.* 1933] to the preparation of bios from malt combings. These fractions had already been assayed for their effects on the respiration of yeast and of rat skin and liver [Cook *et al.* 1938]. In this paper we wish to report on the ultraviolet absorption spectra of these fractions and the activities of four of them on the growth of yeast.

Experimental methods

The preparation and respiration assay of the fractions have been fully reported [Cook *et al.* 1938]. For the growth assays the following fractions were available:

Fraction N: $Ba(OH)_2$–alcohol precipitate.

Fraction C: $Ba(OH)_2$–alcohol filtrate.

Fraction O: Acetone precipitate.

Fraction E: Acetone filtrate.

Unfortunately no more of the original crude aqueous-alcoholic extract (fraction A), from which these samples were prepared, was obtainable for growth

assay although some spectra were taken of this fraction. Other crude extracts comparable with fraction A possess considerable growth activity but, of course, a significant quantitative comparison cannot be made with the fractions used in the present study.

Growth assay. These fractions were tested for their effect on the proliferation of *Saccharomyces cerevisiae* in Reader's medium [Reader, 1927] by the rocker-tube technique previously employed in these laboratories [Norris & Hart, 1937; Norris & Kreke, 1937; Loofbourow, Cook & Stimson, 1938; Loofbourow, Dwyer & Morgan, 1938; also Fraser, 1921; Lucas, 1924]. Each tube contained 24 ml. of Reader's medium and 1 ml. of added material to be assayed. The yeast used for seeding was washed with Reader's medium from 48 hr. slants on Saboraud's medium and added to the rocker-tubes to give a concentration of 0·064 mg./ml. The concentration of yeast used for seeding and the crop after 24 hr. at 30° were determined by means of a photoelectric densitometer. Since Loofbourow & Dwyer [1938] have shown that greater consistency is obtained when the yeast population increase is expressed in terms of weight rather than in terms of count, we have adopted their procedure. Haemocytometer counts reveal that the increase in weight due to the addition of the fractions studied is the result of increased cell number and not merely of increase in cell size.

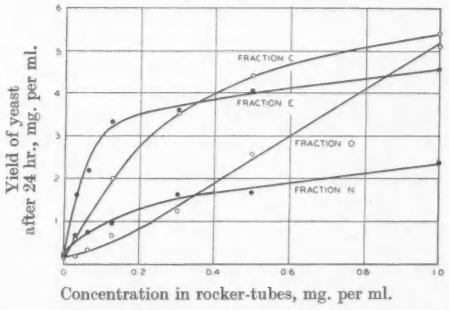

Fig. 1. Stimulating effect of yeast fractions on proliferation of yeast.

The activities of these fractions on the proliferation of yeast are shown in Fig. 1. From these curves have been calculated the potency of the samples in growth units per mg., one growth unit, as defined by Loofbourow, Dwyer & Morgan [1938], representing an increase in yeast crop of 1·6 mg./ml. greater than that in the controls. The growth curves can be regarded as approximately linear up to 0·15–0·25 mg./ml. of added material (except O, which, within experimental error, is linear over its entire length). Beyond this point there is a marked change of slope. The growth unit potency has been calculated within the early portions of the curves. It will be noted that the relative potencies obtained from these parts of the curves usually do not hold when higher concentrations of added material (0·5–1·0 mg./ml.) are considered. Therefore this means of comparing growth potencies may be misleading and, in fact, is really valid only when the growth curves are linear over their entire length. This fact must be borne in mind throughout the discussion. The total number of growth units per fraction, obtained by multiplying the weight of the fraction by the potency in growth units per mg., as well as the potency in growth units per g. of original yeast, have also been calculated. These figures are collected in Table 1. The

Table 1. *Proliferation activity of fractions from yeast*

Fraction	Total wt. of fraction, g.	Growth units per mg.	Total growth units per fraction	Growth units per g. of yeast
N	2·42	2·6	6,292	4
C	41·33	9·1	376,103	237
O	28·28	2·9	82,012	55
E	12·80	22·2	284,160	179
Irradiated yeast preparation 4/26/38*		5·0		715
Sample 14 from malt combings†		10·6		—

* Loofbourow, Dwyer & Morgan [1938]. † Norris & Kreke [1937].

weights of fractions and yields on the basis of yeast were obtained from the previously published data [Cook *et al.* 1938]. Also included for comparison are data on Norris & Kreke's [1937] most potent bios sample from malt combings and the most potent crude material prepared by irradiating yeast [Loofbourow, Dwyer & Morgan, 1938]. With respect to the latter it will be observed that, while its potency in growth units per mg. is considerably lower than some of the preparations obtained without irradiation, the yield per g. of yeast is considerably higher. Irradiated yeast preparations partially purified by chromatographic adsorption show greater potency than any of the bios samples (unpublished data).

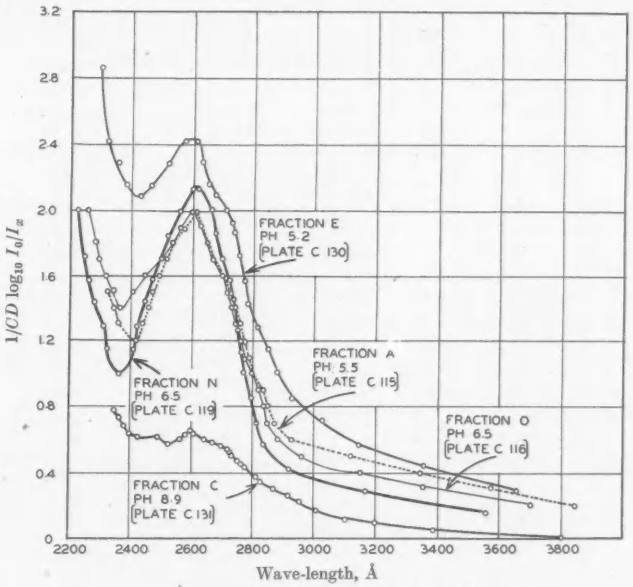

Fig. 2. Ultraviolet absorption spectra of fractions from yeast.

Ultraviolet absorption spectra. Spectra were observed at the normal *p*H of the solutions of the fractions in distilled water. As will be seen from Fig. 2, the reaction varied, being acid in all cases except fraction C. Some months later, in an

attempt to explain certain of the anomalies to be discussed, spectra were re-observed in Kolthoff buffer mixture at *p*H 7. Only slight differences were found in the extinctions with the exception of an increase for fraction N which requires further investigation. The spectra were obtained with a Hilger quartz spectrograph and Spekker photometer using a tungsten spark source and are shown in Fig. 2.

Discussion

The fractionation [Cook *et al.* 1938] employed to obtain the materials studied in this paper involved the extraction of yeast with aqueous alcohol. This crude extract, of which no sample was available for growth assay, was treated with $Ba(OH)_2$ and alcohol. A small amount of precipitate, fraction N (which corresponds to the Bios I fraction of Lucas), contained only a relatively small growth activity, most of the biological activity going into the filtrate (fraction C). This filtrate was then treated for 72 hr. with sufficient acetone to give a concentration of 95 %. This treatment precipitated a considerable quantity of material of relatively low growth potency (fraction O) and the major portion of the growth activity went into the acetone-soluble portion (fraction E), which had a potency of 22 growth units per mg. This fraction would correspond to Lucas's Bios II. There was little loss of potency during the acetone treatment, the sum of the potencies of the acetone filtrate and precipitate being nearly equal to that of the parent fraction C. It is to be presumed that an appropriate variation of conditions would allow a complete concentration of the growth activity in the acetone filtrate and thus permit the removal of a large amount of inactive acetone-insoluble material. As far as the present data go we may say, then, that the yeast growth factor is not precipitated to any great extent by $Ba(OH)_2$-alcohol and is soluble in 95 % acetone.

Reference to the paper on respiration [Cook *et al.* 1938] will show that the disposition of the yeast respiratory activity parallels that of the yeast proliferation activity. In a concentration of 1 mg./ml., fraction C caused a 21 % stimulation of yeast respiration, fraction E a 53 % increase and fraction O was inactive. The close parallelism is shown by the fact that fraction E was 2·5 times as active as C in stimulating respiration and 2·4 times as active in stimulating proliferation. The recovery of respiratory activity (i.e. the sum of E and O as compared with C) was not as complete as in the case of growth. Fraction N, which has a low growth activity, was without influence on yeast respiration. However, both fractions N and O were of considerable activity on tissue respiration (rat liver and skin).

This parallelism of growth and respiratory activity is apparently not in accord with other experiments in which the maximum growth and respiration activities have been concentrated in different fractions [Norris & Hart, 1937; Norris & Kreke, 1937; Norris & Ruddy, 1937]. It was found previously by application of the Lucas fractionation to malt combings [Norris & Kreke, 1937] that the growth activity was concentrated in the acetone precipitate (10·6 growth units per mg.) rather than in the filtrate. On examining the procedures, however, it will be seen that the acetone precipitation as applied to malt combings was a very rapid one, lasting only a few minutes, whereas a 72 hr. acetone treatment was employed with yeast. If we assume that the growth factors from the two sources are the same, which is not necessarily the case, it is readily seen that the prolonged acetone treatment might redissolve material carried down by rapid addition of acetone. In other words, the acetone treatment of Norris & Kreke was inadequate to obtain complete separation. Furthermore, it will be seen that malt combings fractions 18 and 19 [see Norris & Kreke, 1937], which were

obtained from the acetone-soluble material, both possessed high growth activity (6·3 and 5·9 units per mg., respectively) and one of these (no. 18) possessed the greatest respiratory activity of any of the malt combings samples. This incomplete fractionation is significant in view of our present findings. On account of the difference in techniques it is doubtful how far we are justified in comparing the two procedures. While the present experiments show apparent departures from some expectations based on earlier work, the bulk of the evidence at present available still seems to indicate the non-identity of the factors which increase yeast proliferation and respiration. Indeed, current experiments using electrolytic methods favour this non-identity [Cook *et al.*, unpublished]. However, further investigations are certainly required. It is apparent that the classical bios fractionations are not adequate to accomplish a good separation of growth and respiratory factors and results obtained with these methods must be interpreted with care.

All of the fractions show ultraviolet absorption peaks at 2600 Å. although the extinction is very low for fraction C. Absorption at this wave-length is characteristic of the heterocyclic rings of nucleic acids and their derivatives [Holiday, 1930; Heyroth & Loofbourow, 1934]. In keeping with this fact all of the fractions contain N, P and pentose (Bial test). Sulphur is absent, as is protein (biuret test). With the Thomas [1931] tryptophan reagent all fractions gave a light violet-brown colour quite distinct from the pale green given by yeast nucleic and adenylic acids but somewhat resembling the colours given by irradiated yeast preparations and coenzyme II [Cook *et al.* 1939; Loofbourow, Cook & Stimson, 1938]. With the Thomas [1931] β-naphthol reagent the fractions gave green rings with brown tops while yeast nucleic acid gives a blue ring with a green top. The brown coloration was absent from fraction C which thus bore a somewhat closer resemblance to yeast nucleic acid. Irradiated yeast preparations, yeast adenylic acid and coenzyme II gave blue rings with the β-naphthol reagent [Cook *et al.* 1939; Loofbourow, Cook & Stimson, 1938].

An examination of the absorption spectra and growth data appears to show no correlation between the yeast growth activity and absorption at 2600 Å. Thus, fraction N, of low yeast growth potency, has a high absorption. Fraction C has low absorption, but when it is split into its relatively inactive component (O) and its highly active component (E), both of these moieties show greatly increased absorption of the same general magnitude. It seems difficult to attribute the low absorption of C to the presence of non-absorbing materials which are not present in the component fractions O and E because recovery was practically quantitative on a weight and nearly so on a biological basis. The apparently anomalous relation between the spectra of C and its components O and E must go unexplained for the present. If we omit C as such and consider it only in terms of its components O and E, fairly good checks can be obtained between the spectra N, O, and E and the original A. This is shown in Table 2 in which the

Table 2. *Weights of samples and extinctions at 2600 Å.*

Sample	Wt. in g.	Extinction at 2600 Å.	Wt. × Extinct.	$\frac{\text{Wt.} \times \text{Extinct.}}{\text{Wt.}}$
A	102·6	2·00	205·2	2·00
N	2·4	2·14	5·1	—
O	28·3	2·00	56·6	2·13
E	12·8	2·42	31·0	—

extinctions at 2600 Å. and the weights of the fractions are compared. It should also be noted that all fractions show considerable short-wave absorption, as is

true of the preparations from irradiated yeast and tissues. At this time it is not possible to say to what extent this may be connected with the biological activity but this point is under investigation.

From the facts presented it appears that the bios preparations studied may contain variable quantities of material absorbing at 2600 Å. and the biological activities of different partially purified bios fractions cannot be correlated quantitatively with the material having these absorption properties. None of the known pure bios components gives this type of absorption, with the apparent exception of Rainbow & Bishop's [1939] Bios II B. On the other hand, injury to cells seems to result in the production by the cells of proliferation-promoting substances which, at least in the crude state, have characteristic absorption at 2600 Å., the amount of this absorption paralleling the growth potency. It would thus seem possible that a more or less specific type of proliferant may be produced by cells as a result of injury. In this connexion it may be pointed out that materials from injured cells have usually given a linear relationship between growth and concentration over a wide range [Loofbourow, Dwyer & Morgan, 1938; Loofbourow *et al.* 1940]; whereas the growth curves of the bios preparations are usually not linear over their entire length, showing marked stimulation at low concentrations but little greater effect at high concentrations [cf. Norris & Kreke, 1937]. At this time it cannot be certain to what extent this distinction between "bios type" and "injury type" proliferants may exist, especially since current experiments in these laboratories [Loofbourow *et al.* unpublished] show that by chromatographic adsorption it is possible to separate from the materials produced by injured cells very potent proliferation stimulants which do not show selective absorption at 2600 Å. It is certain that injury results in the increased release of materials having these absorption characteristics, but further work alone will show to what extent injury causes cells to produce specific proliferants and to what extent the proliferants may be of the "normal" bios type.

Summary

The yeast proliferation-promoting activity of fractions obtained from yeast is concentrated in a 95% acetone-soluble fraction corresponding to Lucas's Bios II from malt combings. The greatest activity on yeast respiration is also found here. Unlike the crude cell-free preparations from ultraviolet-injured yeast cells, no direct correlation is found between ultraviolet absorption at 2600 Å. and the proliferation activity of the present fractions. This and dissimilar characteristics of the growth curves may indicate a difference in the nature of proliferants produced as a response to injury (intercellular wound hormones) and the known members of the bios complex, or may indicate that a predomination of certain proliferants is produced as a response to injury.

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190. GROWTH FACTOR AND OTHER NUTRITIONAL REQUIREMENTS OF THE ACETONE BUTANOL ORGANISM, *CL. ACETOBUTYLICUM*[1]

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(*Received 26 October 1940*)

THIS investigation has been mainly concerned with the following fundamental problem: What is the simplest possible medium in which normal growth of *Cl. acetobutylicum* will take place? The recent observations of Woolley *et al.* [1939] and of Snell & Williams [1939] have shown that the related organism, *Cl. butylicum*, will grow normally on a synthetic medium containing glucose, in which asparagine is the sole source of nitrogen and to which exceedingly minute amounts of pure biotin have been added. Weizmann & Rosenfeld [1939] have already reported that biotin is a growth factor for *Cl. acetobutylicum*, which differs from *Cl. butylicum* mainly in being proteolytic and in yielding acetone as a fermentation product in place of *iso*propyl alcohol. These authors, however, did not use pure biotin, but concentrates rich in that growth factor obtained from egg yolk (by following the fractionation procedure of Kögl & Tönnis [1936] up to a certain point) and from various plant materials.

Thanks to the great generosity of Prof. Kögl of Utrecht, we have had at our disposal a small specimen of nearly pure biotin and have been able to confirm the general conclusion reached by Weizmann & Rosenfeld [1939], but not the particular one they claimed to have established, which was, to quote their own words, that "the second factor, biotin, together with the first factor, asparagine, brings the specific nutritive level up to 70% as compared with maize". In our experience, *Cl. acetobutylicum* will show practically no growth in a synthetic medium containing asparagine and biotin and no other growth factors. Another factor, which we propose to call the BY factor, not identical with any known factor for micro-organisms, is absolutely essential for growth, while asparagine is not essential. In a medium with glucose as the sole source of carbon and the ammonium ion (supplied as ammonium phosphate) as the sole source of nitrogen, normal growth will take place provided that adequate amounts of both biotin and the BY factor are supplied. Neither factor will support growth in the complete absence of the other.

Yeast extract is particularly rich in the BY factor which has been obtained in a very concentrated form, but not yet in a pure state, from this material. Our best concentrate will enable marked growth of the organism to take place, in presence of excess of biotin, when supplied at the low concentration of 0·025 μg./ml. The biotin requirement for a similar result in presence of excess of the BY factor is even smaller and is of the same order as the known requirement

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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by *Cl. butylicum*. The chemical properties of the BY factor are on the whole very similar to those of biotin; it is a weak acid, very stable to the action of acids and alkalis and is destroyed by nitrous acid.

As already stated, the nitrogen requirements of the organism in presence of the growth factors are very simple. Ammonium phosphate is in fact more suitable than asparagine, or asparagine and ammonium salts, in that growth begins after 28–30 hr., whereas on the asparagine basal medium growth is usually delayed until the third day. This long lag period can be considerably shortened in a number of ways:

(*a*) by addition of hydrolysed casein, or an artificial amino-acid mixture simulating hydrolysed casein;

(*b*) by addition of suitable mixtures of a few amino-acids, valine plus methionine being especially good in this respect;

(*c*) by addition of cysteine or Na_2S.

Finally, some preliminary work has been carried out on the fermentation of glucose in the simplest possible medium containing ammonium phosphate as the sole source of nitrogen and minute but adequate amounts of the two highly purified growth factors. Although the production of solvents does occur, the yield is not as high as in a "normal" fermentation, and the titratable acidity at the end is correspondingly higher. An impure biotin preparation obtained from cane molasses is better in this respect than pure biotin. The reason for this is at present unknown. Most of the common mono- and di-saccharides, as well as starch and mannitol, can also be utilized for growth and solvent production on the above virtually synthetic medium. We have also satisfied ourselves that *Cl. acetobutylicum* can be propagated indefinitely on such a medium; in other words, no unsuspected growth factor was carried over in the initial inoculum. We cannot at the moment offer any explanation of the claim of Brown *et al.* [1939] that the organism can be propagated indefinitely on a glucose–amino-acid–ammonium sulphate–salts medium, save that their amino-acid mixture, which was not exclusively composed of synthetic acids, must have contained both biotin and the BY factor.

Experimental

Cultures and medium. Unless otherwise stated *Cl. acetobutylicum* Strain 5 was used throughout this investigation. This is a strain that has been used in the commercial production of acetone and butyl alcohol. The basal medium most commonly employed has the following composition: 2% glucose (C.P.); 0·1% asparagine; 0·1% $(NH_4)_2SO_4$; 0·11% Speakman's salt mixture (KH_2PO_4; K_2HPO_4; Mg, Mn and ferrous sulphates); reduced iron (at the bottom of the tube). Each tube contained 35 ml. of medium and was incubated at 37° in a closed jar made anaerobic by moistened oats. The standard inoculum was prepared from a 2-day culture which had been inoculated with a spore suspension on soil and heated at 100° for 2 min. before incubation. The medium we used for growing the inoculum was similar to the above, but in it the $(NH_4)_2SO_4$ was replaced by 0·52% peptone. A portion of the culture was centrifuged, the supernatant liquid poured off and the deposit resuspended in an equal volume of sterile distilled water. 0·3–0·4 ml. of this suspension was used as the inoculum. After 3 days' incubation the gas in the tubes was removed by suction and the turbidity read in the Evelyn colorimeter. The reading is inversely proportional to the turbidity or amount of growth. A reading of 100 denotes no growth. "Pure biotin" refers to the crystalline preparation received from Prof. Kögl, which he designated as about 75% pure biotin methyl ester.

Biotin as growth factor for Cl. acetobutylicum. No growth of the organism took place in a 2% glucose–0·1% asparagine–Speakman's salts–basal medium containing pure biotin in a concentration much higher than necessary for optimum growth of the related organism, *Cl. butylicum*, Strain 21 [cf. Peterson *et al.* 1940]. The addition of a mixture of growth factors known to be required by certain micro-organisms (riboflavin, pantothenic acid, nicotinic acid, vitamin B_6, inositol, pimelic acid, uracil, glutathione, vitamin B_1, β-alanine and biotin) gave a similar negative result, as did a mixture of two pyrimidines (uracil and cytosine) and four purines (guanine, adenine, xanthine and hypoxanthine). A biotin-free preparation (*Cl. butylicum* assay) was, however, obtained from yeast extract which alone did not support the growth of *Cl. acetobutylicum*, but when added in conjunction with pure biotin enabled the organism to grow normally as shown by turbidity measurements in the colorimeter.

This biotin-free preparation (referred to subsequently as yeast extract preparation) was obtained as a by-product in the concentration of the second BY factor described later. Difco yeast extract was extracted for 30 hr. with absolute alcohol in a Soxhlet apparatus. The cold extract was filtered, evaporated to dryness *in vacuo* and taken up with cold water. The small amount of residual insoluble gum was extracted with 1000 parts of boiling water, and the filtered extract, after evaporation to dryness, was again taken up in hot water and filtered. The clear neutral solution so obtained contained approximately 50 μg. of dissolved matter per ml. and was essentially free from biotin (see Table 1). Table 1 further shows the response of *Cl. acetobutylicum* to an excess of added biotin in presence of an excess of the yeast extract preparation after incubation at 37° for 3 days.

Table 1. *Biotin as growth factor for butyl organisms*

Organisms and supplement	Supplement μg./ml. Tube 1	Supplement μg./ml. Tube 2	Colorimeter readings Tube 1	Colorimeter readings Tube 2
I. *Cl. butylicum*:				
(1) +pure biotin	0	0·00023	91·5	22
(2) +yeast extract preparation	5	7	85·7	89
II. *Cl. acetobutylicum*:				
(1) +pure biotin	0	0·00023	92	98
(2) +yeast extract preparation	5	7	85	83·5
(3) +pure biotin and yeast extract preparation	0·00023 + 5	—	22	—

Table 2. *Response of* Cl. acetobutylicum *to varying amounts of biotin*

Yeast extract preparation added in excess (5 μg./ml.)

Pure biotin added μg./ml.	Reading	Gas production
0·0000055	66·5	Very slight
0·000011	65	Slight
0·000028	53	Moderate
0·00011	32·5	Good
0·00023	31	Very good

Table 3. *Response of* Cl. acetobutylicum *to varying amounts of the BY factor*

Each tube contained 1 mg. of molasses concentrate as source of biotin

Yeast extract preparation μg./ml.	Reading
0	96
0·7	80
2·8	37·5
5·6	29

Table 2 shows the response of the latter organism to varying amounts of biotin in presence of an excess of the yeast extract preparation, while Table 3 shows the converse, viz. the growth response to varying amounts of the yeast extract preparation in presence of an excess of biotin, supplied in this instance as a concentrate obtained from cane molasses by extraction with cold ethyl acetate, evaporation of the extract to small volume and subsequent aqueous extraction of the filtered extract. The biotin preparation, which was virtually free from the BY factor, was the brown gum left on evaporation of this aqueous extract and is subsequently referred to as "molasses concentrate". By assay against *Cl. butylicum* it was estimated to contain 0·035 μg. of biotin per mg. In order to conserve the supply of pure biotin, the above molasses concentrate was used as a source of biotin wherever possible.

Evidence that biotin and the BY factor are the only growth factors required

The following experiments showed that the yeast extract preparation did indeed contain all the other necessary factors and that there was no carry-over of growth factors from the inoculum. A standard inoculum was made in the following medium: 2 % glucose, 0·1 % asparagine, 0·1 % $(NH_4)_2SO_4$, Speakman's nutrient salts, synthetic *dl*-valine (9 μg./ml.), yeast extract preparation (5 μg./ml.), pure biotin (0·00023 μg./ml.). After 42 hr. the culture showed a good turbidity; it was centrifuged, the supernatant was decanted off completely and the hard deposit of cells was resuspended in an equal volume of sterile distilled water. 0·4 ml. of this suspension was then used as an inoculum for a second tube of medium (35 ml.) of identical composition. This culture showed good growth after 2 days. After another such transfer two more were made into a similar medium but without valine. A sixth transfer was finally made into three separate tubes of the same basal medium without valine:

(*a*) with biotin but with no yeast extract preparation,
(*b*) without biotin but with yeast extract preparation,
(*c*) with both biotin and yeast extract preparation.

Tube (*c*) showed good growth after 36 hr. and gave a reading of 21 after 3 days, whereas the corresponding final readings with (*a*) and (*b*) were 100 and 83·5 respectively. There can be little doubt therefore that the yeast extract preparation contains all the factors other than biotin which are necessary for growth. Three other strains of *Cl. acetobutylicum*, viz. American Type Culture Strains 862 (Kluyver's *Cl. acetonigenus*) and 824 (Rettger's strain), and Weizmann's original strain, responded exactly as did the strain 5. In every case there was no growth on the medium containing only biotin, minimal growth on the medium containing only yeast extract preparation and good growth and gas production in 3 days on the medium containing both growth factors.

Purification and properties of the BY factor

No evidence has yet been secured that the active biotin-free preparations obtained from yeast extract contain more than one growth factor for *Cl. acetobutylicum*. A considerable concentration of this factor has been achieved by following the scheme outlined below, which, in essence, consists of a preliminary concentration of active material by alcohol extraction of Difco yeast extract, followed by a separation from amino-acids by ether extraction at *p*H 1 and then a separation from the stronger organic acids by ether extraction at *p*H 7. Our unit for BY factor activity is that amount of material, which, dis-

solved in 1 ml. of the glucose–asparagine–$(NH_4)_2SO_4$ basal medium containing also 29 μg. of molasses concentrate, will give a reading of 60 after 3 days' anaerobic incubation at 37°. This somewhat arbitrarily chosen unit is really more satisfactory than that corresponding to half-maximum growth, owing to the difficulty often experienced in making an even suspension from the ropy bacterial deposit in a culture showing maximum growth.

No claim is made that the values given for the activities of the various fractions are more than rough approximations. There is evidence that the amino-acids present in the cruder fractions, although not growth factors or growth inhibitors in the strict sense, nevertheless have a considerable effect on the growth of the organism. The extent of the purification achieved may be summarized thus: Difco yeast extract has an activity of 1 unit in 20 μg., is rich in amino-acids and also contains biotin; the "ether-soluble *p*H 7 extract" finally obtained has an activity of 1 unit in 0·05 μg., is free from amino-acids and also essentially free from biotin. The purification procedure is given in more detail in Fig. 1.

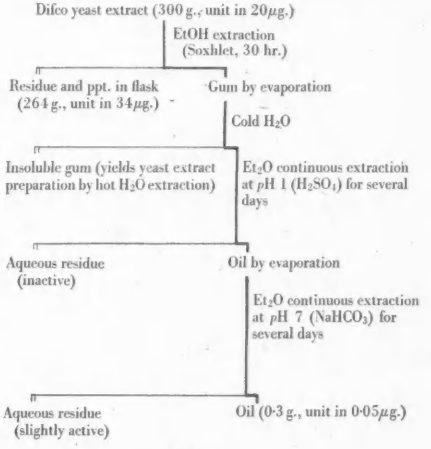
Difco yeast extract (300 g., unit in 20 μg.)
EtOH extraction (Soxhlet, 30 hr.)
Residue and ppt. in flask (264 g., unit in 34 μg.)
Gum by evaporation
Cold H2O
Insoluble gum (yields yeast extract preparation by hot H2O extraction)
Et2O continuous extraction at pH 1 (H2SO4) for several days
Aqueous residue (inactive)
Oil by evaporation
Et2O continuous extraction at pH 7 (NaHCO3) for several days
Aqueous residue (slightly active)
Oil (0·3 g., unit in 0·05 μg.)

Fig. 1.

A fair proportion of relatively inactive material can be removed from this preparation by extraction with an excess of boiling light petroleum, but a more promising mode of purification is to remove coloured impurities by precipitation with phosphotungstic acid and to extract the colourless material recovered from the phosphotungstic acid filtrate with boiling alcohol. Before the alcohol treatment the product contained 1 unit in 0·03 μg. Further work is in progress on the purification of the factor. A brief survey was made of various sources of the factor. Difco yeast extract was the best source, while bakers' yeast, brewers' yeast, alcoholic extracts of corn and bran, distillers' solubles and liver were also quite active. Casein, dried whey, skim milk powder, meat scrap meal and molasses contained little activity.

The factor is adsorbed on norite at *p*H 4 and may be eluted by acetone-ammonia, or alcohol-ammonia. It is not eluted by 50% alcohol. It is not adsorbed on permutit, Lloyd's reagent, or "filtercel". The factor is stable to

$2N$ NaOH or $2N$ H_2SO_4 for 10 min. at 100° and to 30 min. autoclaving at neutrality at 15 lb. pressure. Bromination in chloroform at 25° inactivates it; biotin is not destroyed by this treatment. The factor is destroyed by cold alkaline $KMnO_4$ and by HNO_2. At least 90 % of it is destroyed by 10 min. boiling with excess H_2O_2. It may be noted here that we have been unable to obtain active extracts from hydrolysed casein with unpurified ether, but extracts made with peroxide-free solvent have shown definite, though weak, activity. The BY factor, in contrast to biotin, is completely extracted from yeast by autoclaving with 50 volumes of water. Autolysis gives no increase. The factor is apparently not precipitated by neutral lead acetate, but it has been difficult to recover more than 50 % of the original activity in the filtrate.

Suitability of various simple nitrogenous nutrients in presence of both growth factors

In the experiments described below, the source of biotin was "pure biotin", and the source of the BY factor was the material obtained by a boiling chloroform extraction of the ether-soluble *p*H 7 extract which had previously been extracted with boiling light petroleum and with boiling benzene. It contained 1 unit in 0·05 μg. and was free from biotin.

The basal medium for *Cl. acetobutylicum* previously employed contained both asparagine and $(NH_4)_2SO_4$ as sources of nitrogen. The latter was added because in its presence the maximum growth attained with both growth factors in excess seemed to be a little heavier than in the basal medium containing asparagine as sole source of nitrogen. In neither case however did growth begin until the end of the 2nd day, often not until the 3rd day. A basal medium containing $(NH_4)_2SO_4$ as sole source of nitrogen was found to be even less suitable, but a remarkable improvement was effected by using ammonium phosphate (0·1 %) as the sole source of nitrogen, the initial *p*H of the medium being 6·3–6·5. In this medium growth invariably began after 28–30 hr., sometimes sooner, and the maximum growth attained, in the presence of excess of the growth factors, was practically the same as in the asparagine–$(NH_4)_2SO_4$ basal medium. Table 4 gives the results of one such comparison made between the two basal media. All the tubes were incubated in the same anaerobic jar and each tube contained 1·1 μg. of the BY factor per ml. The ammonium phosphate medium contained $(NH_4)_2HPO_4$ (0·1 %) adjusted to *p*H 6·5 by addition of the requisite amount of H_3PO_4.

Table 4. *Comparison of nitrogen sources*

	NH_4 phosphate basal			Asparagine-$(NH_4)_2SO_4$ basal		
Biotin added μg./ml.	40 hr.	64 hr.	Reading after 5 days	40 hr.	64 hr.	Reading after 5 days
0	–	–	90	–	–	93
0·00006	±	+	80	–	±	73
0·00012	±	+	78	–	±	65
0·0007	+ +	+ + +	61	–	±	39
0·0014	+ +	+ + +	30	–	+ +	38

Although the final response to amounts of biotin insufficient for maximum growth is poorer on the ammonium phosphate medium, it is equally good when excess of biotin is present, and in all instances growth begins about a day earlier on this medium. It is a singular fact that an asparagine-ammonium phosphate

medium shows but little improvement over the customary asparagine-ammonium sulphate basal medium in this respect. It is therefore justifiable to conclude that although *Cl. acetobutylicum* is undoubtedly a proteolytic organism it can make, and perhaps prefers to make, all the amino-acids it requires from the glucose and the ammonium ion, in this respect resembling other proteolytic organisms which are not strict anaerobes, e.g. *Ps. fluorescens* and *Pr. vulgaris*. It is also noteworthy that filtrates from these last-named organisms, grown in a purely synthetic glucose-NH_4Cl medium, undoubtedly contained relatively large amounts of both biotin and the BY factor. On the other hand, *Cl. acetobutylicum*, when grown in a medium containing an excess of both growth factors, seems to remove them from solution almost entirely.

It may be mentioned that when biotin is supplied as molasses concentrate, the superiority of the ammonium phosphate basal medium is much less marked, since growth begins much earlier in the asparagine-$(NH_4)_2SO_4$ tubes than when biotin is supplied in the form of pure biotin.

Effect of various sulphur-containing and iron-containing compounds on the growth of the organism in simple media

The ensuing remarks refer only to growth in the asparagine-$(NH_4)_2SO_4$ basal medium containing excess of pure biotin and the BY factor as on page 1590. It has been found that the addition of small amounts of certain reducing agents, or the oxidized form of these substances, enables growth to begin much sooner than normally. The following is a list of the active substances so far encountered and the concentrations at which they have been found to be active:

Cysteine	1–3·5 mg. per tube (but not 10 mg. per tube)
Cystine	Ditto
$Na_2S,9H_2O$	1–4 mg. per tube (but not 6 mg. per tube)
Na hydrosulphite	1 mg. per tube
K ferricyanide	2 mg. per tube

The following have no such effect in speeding up growth: Na_2SO_3, Na thiol-acetate, catehol quinol. NaCN and K ferrocyanide inhibit growth altogether. Of the active substances, cysteine, cystine and Na hydrosulphite seem to be the most effective. The extent to which these compounds shorten the lag phase is evident from Table 5.

Table 5. *Effect of S- and Fe-containing substances on growth*

Addition μg./ml.	Growth after 37 hr.	42 hr.	47 hr.	61 hr.	Final reading after 3·5 days
None	−	−	−	±	26
Cysteine HCl: 29	+	+ +	+ + +	+ + +	22·5
Cysteine HCl: 58	+	+ +	+ + +	+ + +	24·5
Cysteine HCl: 101	+ +	+ + +	+ + +	+ + +	22
Cystine: 29	+	+ +	+ + +	+ + +	30
$Na_2S,9H_2O$: 29	±	.	+ +	.	41·5
$Na_2S,9H_2O$: 58	+	.	+ + +	.	39
$Na_2S,9H_2O$: 87	+	+ +	+ + +	+ + +	43
$Na_2S,9H_2O$: 116	±	±	+ +	+ + +	22
Na hydrosulphite: 29	+	.	+ + +	.	28
$K_3Fe(CN)_6$: 58	−	±	+ +	+ + +	23

The addition of cysteine hydrochloride (29 μg./ml.) and of $Na_2S,9H_2O$ (87 μg./ml.) also hastened growth by a few hours in the ammonium phosphate

basal medium. In fact, tubes containing these additions behaved exactly as a tube containing molasses concentrate (43 μg./ml.) as source of biotin, but no added cysteine or Na_2S. The molasses concentrate gave a negative ninhydrin reaction, but may of course contain sulphur compounds other than amino-acids.

Methionine has a similar, but much weaker, effect than cysteine and cystine. Since it cannot act as a redox agent, it is considered with other amino-acids in the next paragraph.

Effect of amino-acids on the growth of the organism in simple media

It has to be admitted that, even in the ammonium phosphate medium, growth does not begin as soon as when a small amount of ether-extracted hydrolysed casein (equivalent to 500 μg. of casein per ml.) is added in addition to pure biotin and the BY factor concentrate.

A few preliminary observations on the effect of single amino-acids and artificial mixtures of amino-acids on the growth of *Cl. acetobutylicum* in the asparagine-$(NH_4)_2SO_4$ basal medium are here recorded. It has become abundantly evident that the problem is one of great complexity, not only because certain amino-acids seem to be helpful and others antagonistic to growth, but because their effects are markedly different according to whether the growth factors are present in excess or in amounts barely sufficient to support good growth. There is further a surprising lack of parallelism between growth and the utilization of glucose when mixtures of amino-acids are present.

In the first place, an artificial mixture of 19 amino-acids simulating hydrolysed casein [Hutchings & Woolley, 1939] did have much the same effect as ether-extracted hydrolysed casein. When 1 mg. of this mixture was added per tube, growth was evident after only 25 hr. incubation. The mixture contained 8 amino-acids of which optically inactive synthetic specimens were available, and the effects of these acids, viz. glycine, *dl*-alanine, *dl*-valine, *dl*-leucine, *dl*-serine, *dl*-phenylalanine, *dl*-threonine and *dl*-methionine, were next studied. Of these, valine and methionine (particularly when added together) were definitely helpful, and leucine definitely inhibitory to growth. Table 6 brings out these findings very clearly.

Table 6. *Effect of amino-acids on the growth of* Cl. acetobutylicum

Basal medium: 2% glucose–asparagine–$(NH_4)_2SO_4$–Speakman's salts.
Pure biotin = 0·00023 μg./ml.
BY factor = 5 μg. yeast extract preparation per ml.

Addition μg./ml.	Growth after 37 hr.	41 hr.	59 hr.	Reading after 4 days
None	–	–	+ +	31
dl-Valine: 9·2	±	+	+ + +	26
dl-Methionine: 4·6	±	+	+ + +	27
Valine and methionine (as above)	+ +	+ + +	+ + +	29
dl-Leucine: 5·7	–	–	±	33
8 amino-acids: total concentration = 29	+ +	+ + +	+ + +	30

It should be pointed out, however, that the residual glucose after 4 days' incubation was highest in the valine plus methionine tube (0·60%) and lowest in the valine tube (0·08%). Growth and fermentation are two quite distinct activities of the organism, as is shown by the results of the concluding sections.

Fermentation of glucose in the ammonium phosphate basal medium containing the growth factors

Although detailed analyses for individual fermentation products have not been made, the yield of solvents, when biotin is supplied as pure biotin, and the BY factor as a highly active concentrate, approximates to, but does not quite equal, the normal. If biotin is supplied as molasses concentrate the titratable acidity at the end of the fermentation is invariably lower than when pure biotin, in amounts equal to or greater than those present in the molasses concentrate, is supplied in its stead (see Table 7). The yield of acetone is usually 4–6% of the sugar fermented, and, given sufficient biotin, the organism will completely or almost completely ferment 2% glucose in the medium. It has to be remembered that the BY factor is supplied in an impure form, and the possibility certainly exists that a third "accessory" factor may be required for normal fermentation as distinct from normal growth. Further work on this aspect of the problem is in progress. The ash obtained on ignition of the molasses concentrate has no activity as an accessory factor.

Table 7. *Fermentation of glucose on a virtually synthetic medium*

BY factor supplied as 1·1 μg. of concentrate per ml.

No. of exp. and period of incubation days	Biotin, source and amount μg./ml.	Initial conc. glucose %	Final conc. glucose %	Titratable acidity, ml. 0·1 *N* NaOH per 10 ml. of medium
I. 3·5	Pure biotin: 0·003	2·0	Nil	6·4
	Molasses concentrate: 29	2·0	Nil	3·1
II. 2·5	Pure biotin: 0·006	2·0	0·5	3·7
	Molasses concentrate: 29	2·0	0·5	2·7
III. 3·5	Pure biotin: 0·006	2·0	0·1	3·0
	Molasses concentrate: 29	2·0	0·1	2·4
IV. 5·5	Pure biotin: 0·006	3·0	0·8	4·0
	Molasses concentrate: 29	3·0	0·4	2·5

Sugars fermented by Cl. acetobutylicum *on a virtually synthetic medium containing ammonium phosphate as the only source of nitrogen*

In the presence of pure biotin and a highly active concentrate of the BY factor, the following were fermented: glucose, galactose, mannose, fructose, xylose, *l*-arabinose, sucrose, lactose, cellobiose, maltose, mannitol, starch, salicin. The lag period, the yield of acetone and the final acidity showed considerable variations from sugar to sugar. No growth took place on the following: glycerol, dulcitol, sorbitol, sorbose, *d*-arabinose, rhamnose, trehalose, heptose, inulin, lactic acid and pyruvic acid. Further work along these lines is in progress.

SUMMARY

Two growth factors are required by *Cl. acetobutylicum*, viz. biotin and another factor, present in yeast extract, which is not identical with any known growth factor for micro-organisms, and which has been obtained in a highly concentrated form. In presence of these two factors the organism will grow normally and also produce solvents from glucose with ammonium phosphate as

the sole source of nitrogen. There is no strict correlation between growth and fermentation, and the presence of other factors may be necessary in order that normal solvent production may take place concurrently with normal growth. Certain other sugars are also fermented in the simple ammonium phosphate medium containing the two growth factors.

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191. VITAMIN B_1 AND THE SYNTHESIS OF OXALOACETATE BY *STAPHYLOCOCCUS*

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(*Received 29 October 1940*)

It has been suggested by Krebs & Eggleston [1940, 1] that the function of vitamin B_1 in pigeon liver is to catalyse the formation of oxaloacetate from pyruvate and CO_2:

$$CH_3.CO.COOH + CO_2 \xrightarrow{\text{vitamin } B_1} COOH.CO.CH_3.COOH. \qquad (1)$$

The present work was undertaken to see if there is evidence for a similar mode of action of vitamin B_1 in the metabolism of *Staphylococcus*.

Staphylococci and other bacteria convert pyruvate anaerobically into lactate, acetate and CO_2 [Krebs, 1937]:

$$2CH_3.CO.COOH + H_2O \rightarrow CH_3.CHOH.COOH + CH_3.COOH + CO_2. \qquad (2)$$

Hills [1938] found that this dismutation depends on the presence of vitamin B_1. In organisms grown on vitamin-deficient media the rate of dismutation is much reduced, and addition of vitamin B_1 restores the rate to its normal level. How reaction (1) is related to the dismutation of pyruvate is not at first sight obvious, but if it is assumed (*a*) that oxaloacetate acts as a carrier in reaction (2), (*b*) that oxaloacetate is formed by reaction (1), then the action of vitamin B_1 in *Staphylococcus* can be explained in the same way as in pigeon liver. The results described here are in agreement with assumptions (*a*) and (*b*) and support the view that in *Staphylococcus*, as in pigeon liver, vitamin B_1 catalyses the synthesis of oxaloacetate from pyruvate and CO_2.

Experimental methods

S. albus was used in most of the experiments, but the main reactions were also tested with *S. aureus*. Cultures of these organisms were obtained from Dr D. Lewis of the Department of Bacteriology of this University. Two types of culture media were used, one containing vitamin B_1 in excess of the amounts required for growth and metabolism, while the other was deficient in vitamin B_1. For the "vitamin-sufficient" cultures a standard medium of this laboratory was used, containing 1% lemco meat extract, 1% peptone (difco), 0·5% yeast extract (difco), 0·5% NaCl, 1% glycerol and 3% agar. The bacteria were incubated aerobically in Roux bottles for 48 hr., washed in 0·9% NaCl 3 times by centrifuging and finally suspended in 0·9% NaCl. The amounts of bacteria in the suspensions were determined by drying and weighing a known volume of the suspension. If not used immediately the suspensions were stored at 0°. The preparation of the "vitamin-deficient" cultures is described in the next section.

The dismutation of pyruvate was studied by shaking suspensions of bacteria in the presence of pyruvate in Warburg manometer cups at 40° and measuring the amount of CO_2 evolved. The cups were filled with 5% CO_2 in N_2, and anaerobic conditions were ensured by the presence of yellow phosphorus in

the centre chamber of the cup. For these experiments the stock bacterial suspensions were diluted with 0·9 % NaCl and 1·3 % bicarbonate so as to give a final bicarbonate concentration of 0·023*M*. This concentration in equilibrium with 5 % CO_2 gives a *p*H of 7·4. Before diluting the bacterial suspension with the bicarbonate saline the latter was equilibrated with 5 % CO_2.

Additions of pyruvate, oxaloacetate and fumarate were made from the side arms of the cups in the form of solutions of the sodium salts. Oxaloacetate was prepared freshly from oxaloacetic acid immediately before use. "Aneurin" (Roche Products) was the preparation of vitamin B_1 used. The estimation of succinate, fumarate and malate was carried out as described in recent papers from this laboratory [Krebs & Eggleston, 1940, 2; Krebs *et al.* 1940].

Preparation of the vitamin-deficient cultures

In the earlier experiments the vitamin-deficient organisms were prepared as described by Hills [1938] on the basal medium of Fildes & Richardson [1937] in which the aneurin was reduced to a concentration of $3–4 \times 10^{-9}M$. Later it was found that vitamin-deficient organisms could be obtained more easily by using a basal medium prepared from casein either by acid hydrolysis or by tryptic digestion [Gladstone & Fildes, 1940]. The casein acid hydrolysate was diluted with 15 volumes, and the tryptic digest with 3 volumes, of phosphate buffer (*p*H 7·4), and sufficient glycerol was added to make a concentration of 2 %. This was used in Roux bottles with 3 % agar for aerobic growth. No vitamin B_1 was added as it was found that this medium contained enough vitamin for growth, but the organisms obtained after 40 hr. growth were found to be vitamin-deficient.

Inoculation was made with a few ml. of vitamin-deficient medium, without agar, which had been inoculated 24 hr. previously with a small platinum loop from a stock culture.

In this paper the vitamin-sufficient medium described above is referred to as the standard medium, the basal medium of Fildes and Richardson as the synthetic vitamin-deficient medium, and the media prepared from casein by acid hydrolysis or tryptic digestion as the casein acid hydrolysate and casein tryptic digest respectively.

As a test of the degree of vitamin deficiency the effect of added aneurin on the rate of dismutation of pyruvate was used. This criterion, used throughout this work, is based on the finding of Hills that the stimulating effect of aneurin on the rate of dismutation occurs only in vitamin-deficient organisms. Table 1 shows the effect of addition of aneurin on the pyruvate dismutation by *S. albus* and *aureus* grown on various media. It is seen that in the case of the organisms grown on the standard medium, addition of aneurin has no effect, whereas there is an increase in the rate with organisms grown on the synthetic medium of Fildes & Richardson, and also with those grown on the casein acid hydrolysate and casein tryptic digest. To show that the difference in behaviour of the bacteria grown on the latter media was due to deficiency of vitamin B_1, cultures were made on media prepared from casein but with aneurin added to a concentration of $3 \times 10^{-7}M$. It is seen that in these organisms the addition of aneurin has no effect on the rate of dismutation of pyruvate.

Attempts were made to obtain vitamin-deficient staphylococci by prolonged washing in saline or distilled water of bacteria grown on the standard medium. The bacteria were suspended in saline or distilled water and repeatedly centrifuged and resuspended. Even after 72 hr. of this treatment there was no evidence of vitamin deficiency as indicated by the above test.

Table 1. *The effect of added aneurin on the rate of dismutation of pyruvate by staphylococci grown on various media*

4 ml. of bacterial suspension; initial concentration of pyruvate 0·025 *M*

Medium	Organism	Amount of bacteria per cup mg.	Added aneurin μg.	CO_2 evolved (μl.) 30 min.	60 min.	120 min.	% increase due to vitamin
Standard	*S. albus*	21	0	191	352	786	
			6·6	184	348	762	−3
Standard	*S. aureus*	9	0	143	260	529	
			20	147	267	559	+6
Synthetic vitamin-deficient	*S. aureus*	4·9	0	15	32	71	
			10	30	65	120	+69
Synthetic vitamin-deficient	*S. albus*	5·7	0	19	40	79	
			10	29	61	123	+56
Casein acid hydrolysate	*S. albus*	6·5	0	54	85	138	
			10	103	202	402	+191
Casein tryptic digest	*S. albus*	7·7	0	62	141	308	
			10	136	369	808	+162
Casein acid hydrolysate with aneurin 3×10^{-7} *M*	*S. albus*	20	0	213	442	915	
			10	221	463	934	+2

It was also thought that the degree of vitamin deficiency of the casein acid hydrolysate or the casein tryptic digest might be increased by treatment with strong alkali. Autoclaving the medium at 125° for 2 hr. with 10 *N* NaOH caused a great decrease in the growth of the cultures, but the resulting organisms did not show any greater degree of vitamin deficiency.

It is evident that for producing vitamin-deficient organisms in large amounts it is simpler to use a casein hydrolysate rather than the synthetic medium of Fildes & Richardson which contains about 25 different constituents which are not all readily available and which require various methods of sterilization.

Effect of oxaloacetate on the dismutation of pyruvate

If the assumptions are true that vitamin B_1 catalyses reaction (1) and that oxaloacetate acts as a carrier in reaction (2), it is to be expected that oxaloacetate added to vitamin-deficient organisms will have a similar effect on the dismutation of pyruvate as aneurin. Comparisons were therefore made of the effects of aneurin and oxaloacetate on the rate of dismutation of pyruvate by vitamin-deficient staphylococci. Fig. 1 shows the results of an experiment of this kind. Addition of aneurin causes a large increase in the rate of pyruvate dismutation. Oxaloacetate causes an even greater initial increase, but the effect falls off, so that after about 60 min. the rate is no greater than in the presence of pyruvate alone. Such a temporary effect is to be expected since oxaloacetate is rapidly decomposed. If oxaloacetate alone is added without pyruvate there is an evolution of CO_2, probably mainly due to the dismutation of pyruvate resulting from the decomposition of oxaloacetate. When oxaloacetate and aneurin are added together in the presence of pyruvate the effect is an initial increase equal to that produced by oxaloacetate alone in the presence of pyruvate and a maintained increase equal to that produced by adding vitamin alone. Thus the effect of added oxaloacetate is not the sum of the effects of oxaloacetate and pyruvate added separately, nor is the effect of oxaloacetate added in addition to aneurin the sum of the effects of each added separately in the presence of pyruvate. The effect of oxaloacetate on the dismutation is not superimposed on that of

aneurin but replaces it. Table 2 summarizes the results of a number of experiments of this type. In all these the addition of oxaloacetate causes an initial increase in the rate greater than that caused by the vitamin. It should be borne in mind that the decomposition of oxaloacetate in itself does not yield free CO_2 but bicarbonate, so that this does not interfere with the measurement of the dismutation of the pyruvate. The evolution of CO_2 in the presence of oxaloacetate alone is due to the dismutation of the pyruvate resulting from the decomposition of the oxaloacetate.

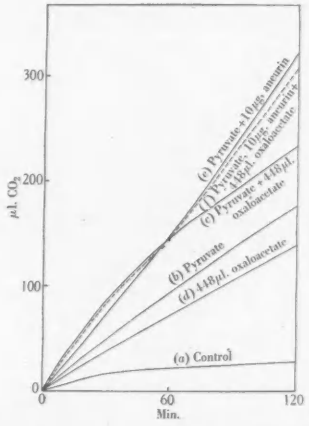

Fig. 1. Effects of aneurin and oxaloacetate on the rate of dismutation of pyruvate by *Staphylococcus albus*. In each cup were 4 ml. of a suspension containing 7·6 mg. of bacteria. Where pyruvate was present the initial concentration was 0·025 *M*. The curves show the evolution of CO_2 (*a*) with no added substrate, (*b*) with pyruvate added, (*c*) with pyruvate and 448 μl. oxaloacetate, (*d*) with 448 μl. oxaloacetate without pyruvate, (*e*) with pyruvate and 10 μg. aneurin, and (*f*) with pyruvate, 10 μg. aneurin and 448 μl. oxaloacetate (interrupted line).

Table 2. *Effects of aneurin and oxaloacetate on the rate of dismutation of pyruvate by vitamin-deficient staphylococci*

4 ml. of bacterial suspension; initial concentration of pyruvate 0·025 *M*

Organism	Medium	Amount of bacteria per cup mg.	Catalyst added	CO_2 evolved (μl.) 10 min.	20 min.	40 min.	60 min.	120 min.	180 min.
S. aureus	Synthetic vitamin-deficient	5·6	—	6	10	19	25	42	54
			Aneurin 20 μg.	16	22	38	51	85	110
			Oxal. 89 μl.	19	24	34	45	63	80
S. aureus	Synthetic vitamin-deficient	4·9	—	5	10	20	32	71	117
			Aneurin 10 μg.	11	24	43	65	135	212
			Oxal. 89 μl.	15	32	58	84	155	212
S. albus	Casein tryptic digest	10·8	—	21	60	139	222	453	686
			Aneurin 10 μg.	52	120	274	440	969	1276
			Oxal. 448 μl.	66	127	230	311	608	852
S. albus	Casein tryptic digest	7·6	—	16	32	65	90	176	266
			Aneurin 10 μg.	22	45	96	144	318	538
			Oxal. 448 μl.	32	58	107	143	233	292
S. albus	Synthetic vitamin-deficient	5·7	—	5	12	26	40	79	114
			Aneurin 10 μg.	7	18	40	61	123	189
			Oxal. 448 μl.	22	40	72	100	156	208

Control experiments with bacteria grown on the standard medium (Table 3) show that the addition of oxaloacetate has no effect on the rate of dismutation in vitamin-sufficient cells.

As a further control cultures were made on the casein acid hydrolysate medium supplemented with $3 \times 10^{-7} M$ aneurin. In organisms grown under these conditions the rate of dismutation was likewise not affected by the addition of oxaloacetate (Table 3).

Table 3. *Effect of oxaloacetate on the rate of dismutation of pyruvate by vitamin-sufficient staphylococci*

4 ml. bacterial suspension; initial concentration of pyruvate 0·025 *M*

Organism	Medium	Amount of bacteria per cup mg.	Oxaloacetate added μl.	CO_2 evolved (μl.) 10 min.	20 min.	40 min.	60 min.	120 min.
S. aureus	Standard	4·6	—	8	16	41	73	169
			89	8	15	38	68	160
S. albus	Standard	8·4	—	57	142	326	371	—
			89	56	149	327	363	—
S. aureus	Standard	5·5	—	22	54	108	164	340
			89	16	41	86	133	271
S. albus	Standard	9·8	—	21	35	61	86	154
			448	24	39	66	92	165
S. albus	Casein acid hydrolysate with aneurin $3 \times 10^{-7} M$	13	—	39	78	151	224	430
			448	37	75	147	216	426
S. albus	Casein acid hydrolysate with aneurin $3 \times 10^{-7} M$	20	—	65	139	291	442	915
			448	68	143	302	463	934

These results are all in keeping with the assumptions that the function of vitamin B_1 in the staphylococcus is concerned with the synthesis of oxaloacetate, and that this substance acts as a catalyst in the dismutation of pyruvate.

Effect of fumarate

A few experiments were done to see if oxaloacetate could be replaced by fumarate as a catalyst in the dismutation. This is to be expected if oxaloacetate acts as a hydrogen carrier in reaction (2). Table 4 shows the effects of fumarate on the dismutation of pyruvate in vitamin-deficient and vitamin-sufficient organisms; it is seen that fumarate acts in a similar way to oxaloacetate, i.e. it increases the rate of dismutation in the vitamin-deficient bacteria.

Formation of succinate, fumarate and malate

In order to obtain some information about the catalytic action of oxaloacetate described in the previous sections, a study was made of the fate of oxaloacetate added to bacterial suspensions. The amount of CO_2 evolved and the amounts of succinate, malate and fumarate formed were measured. Most of the oxaloacetate decomposes to form pyruvate which then undergoes dismutation, the amount of CO_2 evolved being equivalent to about half of the oxaloacetate added. A small fraction is reduced to succinate, fumarate and malate. In one experiment, included in Table 5, the amount of succinate was 1·7 % of the total oxaloacetate added, and the amount of fumarate and malate equal to 14·8 %.

Table 4. *Effect of fumarate on the dismutation of pyruvate by vitamin-sufficient and vitamin-deficient staphylococci*

4 ml. of bacterial suspension; initial concentration of pyruvate 0·025 *M*

Organism	Vitamin content of medium	Amount of bacteria per cup mg.	Added fumarate μl.	CO_2 evolved (μl.) 10 min.	20 min.	30 min.	60 min.	120 min.
S. aureus	Deficient	5·6	—	6	10	19	25	45
			89	19	24	34	45	63
S. albus	Deficient	11·0	—	13	26	50	73	138
			448	18	34	63	93	169
S. albus	Sufficient	7·5	—	77	200	512	801	—
			89	80	186	486	812	—
			448	80	201	472	796	—
S. aureus	Sufficient	10·1	—	36	81	165	253	652
			89	40	87	176	268	561

Table 5. *Formation of succinate, fumarate and malate from oxaloacetate and pyruvate*

4 ml. of bacterial suspension

Organism	Substrate	Succinate μl.	Fumarate and malate μl.
S. aureus	Pyruvate, 2240 μl.	—	91
S. aureus	Pyruvate, 2240 μl.	6	131
S. albus	Pyruvate, 3584 μl.	43	194
S. albus	Pyruvate, 3584 μl.	22	92
S. aureus	Oxaloacetate, 2240 μl.	38	332
S. albus	Oxaloacetate, 2240 μl.	55	—

The assumption that oxaloacetate is synthesized from pyruvate and CO_2 can now be tested further by seeing if the products of reduction of oxaloacetate are found when pyruvate alone is added. Table 5 shows that succinate, fumarate and malate are formed by staphylococci from pyruvate, which suggests that pyruvate is used for the synthesis of oxaloacetate.

Discussion

It has been shown that oxaloacetate or fumarate can replace vitamin B_1 as a catalyst in the dismutation of pyruvate by *Staphylococcus*. This result is explained by assuming that the function of vitamin B_1 is to catalyse the formation of oxaloacetate, and that this substance then acts as a hydrogen carrier. No other suggested function of vitamin B_1 can explain the findings. Of special significance is the fact that the oxaloacetate effect is not obtained in staphylococci grown on vitamin-sufficient media. These results imply that the Szent-Györgyi cycle plays a part in the metabolism of *Staphylococcus*, and they also indicate the manner in which the oxaloacetate is formed in this organism.

The bacterial strains used in these experiments did not exhibit a lag period on addition of pyruvate before the dismutation reached its maximum rate, differing in this respect from those used by Krebs [1937]. In Krebs' experiments the lag period was reduced by fumarate and other substances. In the present work no effects of this kind were obtained in the vitamin-sufficient cells. The causes of these differences have not yet been investigated. One possibility is the difference in the culture media, as in Krebs' experiments no glycerol was present.

SUMMARY

1. *Staphylococcus albus* and *aureus* grown on a casein acid hydrolysate or casein tryptic digest with addition of glycerol were found to be deficient in vitamin B_1. Addition of aneurin caused an increase in the rate of dismutation of pyruvate by washed suspensions of the cells.

2. Aneurin can be replaced for limited periods by oxaloacetate or fumarate. The initial effect of these substances on the rate of pyruvate dismutation is greater than that of aneurin, but it falls off within about 1 hr., while that due to aneurin is steadily maintained.

3. Succinate, fumarate and malate are formed by *Staphylococcus* after the addition of either oxaloacetate or pyruvate.

4. These results support the view that vitamin B_1 catalyses the formation of oxaloacetate, and that this substance acts as a hydrogen carrier in the dismutation of pyruvate.

I wish to thank Dr H. A. Krebs for facilities for carrying out this work, and also for his constant help and advice. The expenses of this work were defrayed by grants to Dr Krebs from the Medical Research Council and the Rockefeller Foundation.

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192. PRODUCTION OF PHOENICINE ON SYNTHETIC MEDIA

1. *PENICILLIUM PHOENICEUM* VAN BEYMA

2. *PENICILLIUM RUBRUM* GRASBERGER-STOLL

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(*Received 28 October 1940*)

1. *PENICILLIUM PHOENICEUM* VAN BEYMA

Penicillium phoeniceum, representative of Thom's classification of *Monoverticillata ramigena*, produces a red pigment, phoenicine, which, being water-soluble, diffuses almost entirely into the medium. Its catalytic function in the respiration of living cells and certain of its properties were first described by Friedheim [1933]. The constitution, viz. 2:2′-dihydroxy-4:4′-ditoluquinone, was established by Posternak [1938].

Friedheim states that phoenicine is produced by old cultures of the organism, grown on beerwort. On this medium the pigment does not appear until after the formation of the green conidia, but a further statement establishes the fact that phoenicine is formed at the outset, when the mould is cultured on media rich in carbohydrates. *P. phoeniceum*, which does not appear to have been previously grown on sugar media, was therefore inoculated on synthetic media containing 5% glucose, of which three were chosen, viz. acid Czapek-Dox, Raulin-Thom and Raulin-Dierckx, a neutral medium.

Experimental

A culture of this penicillium, obtained from the Centraalbureau voor Schimmelcultures, showed conidia formation in 3–4 days and pigment production in 4–5 days.

Nine litres of each of the three media were made up as follows:

Acid Czapek-Dox, p*H* 4·2. Glucose, 50 g.; $NaNO_3$, 2·0 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; KH_2PO_4, 1·0 g.; $FeSO_4$, $7H_2O$, 0·1 g.; distilled water to 1000 ml.

Raulin-Thom, p*H* 3·9. Glucose, 75 g.; tartaric acid, 4·0 g.; NH_4 tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; $MgCO_3$, 0·4 g.; K_2CO_3, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; $FeSO_4$, $7H_2O$, 0·07 g.; $ZnSO_4$, $7H_2O$, 0·07 g.; distilled water to 1500 ml.

Raulin-Dierckx, p*H* 7·0, recommended for the extensive and varying pigment production which it induces among certain penicillia, was employed in a slightly modified form, glucose being used instead of the sucrose, specified by Biourge. Glucose, 46·6 g.; $(NH_4)_2HPO_4$, 0·4 g.; NH_4NO_3, 2·66 g.; K_2CO_3, 0·4 g.; $(NH_4)_2SO_4$, 0·16 g.; $FeSO_4$, $7H_2O$, 0·04 g.; $ZnSO_4$, $7H_2O$, 0·04 g.; 66·7 ml. of a solution of 0·4 g. $MgCO_3$ and 0·71 g. tartaric acid in 100 ml. distilled water added and the volume adjusted to 1000 ml.

The media were distributed in 100 ml. amounts in 20 oz. flat bottles, which were plugged with cottonwool, sterilized by steaming on each of three consecutive days and inoculated with a spore suspension of *P. phoeniceum*, grown for 6–7 days on malt-agar slopes. The bottles were incubated in the dark at 20° for 18 days. Mycelium formation became apparent in 2–3 days, the entire surface of the liquid being overgrown in 5–6 days. At this stage, pigment appeared and was complete throughout by the 8th day, there being no formation of green conidia.

By the 10th day, the mycelium in all three cases had taken on a corrugated appearance. On the Czapek-Dox and Raulin-Thom media, the mycelial felts, upper and reverse sides, were white, while on the Raulin-Dierckx substrate, the felt was light brown, very thin and heavily perforated. Furthermore, this medium was golden yellow throughout almost the entire period of growth, while in both the other cases, the colour varied from shades of red to violet, allowance being made for the intensity of colour due to the increasing concentration of pigment.

*p*H determinations were made at intervals of 3 days, the mixed contents of four bottles being used for each reading. These results are contained in Table 1.

Table 1

Medium ...	Czapek-Dox		Raulin-Thom		Raulin-Dierckx	
Days of incubation	*p*H	Colour of substrate	*p*H	Colour of substrate	*p*H	Colour of substrate
0	4·2	—	3·9	—	7·0	—
3	4·6	—	4·1	—	6·1	—
6	6·7	Violet	4·9	Red	3·9	Red
9	5·1	Red	8·0	Violet	3·3	Orange
12	3·7	Red	6·4	Violet	1·8	Golden yellow
15	3·4	Red	5·4	Red	1·8	Golden yellow
18	3·8	Red	6·6	Violet	1·9	Golden yellow

In correlating these figures, it appears that phoenicine has two colour changes, which may be roughly defined by reference to the *p*H values, yellow to red, *p*H 1·8–3·4, and red to violet, *p*H 5·4–6·4. In addition to the trend of the *p*H figures, the selection of 18 days as the optimum period of growth was supported by the fact that individual bottles, of 16, 17, 19 and 20 days' incubation showed slightly decreased yields of the pigment. There now remained 60 bottles of each medium.

The mycelium was strained off and washed with a small quantity of water, the washings being added to the main bulk of the filtrate. The mycelium, pressed and dried *in vacuo* at 55° was weighed and Soxhlet-extracted with no result of interest. An attempt to concentrate the filtrate *in vacuo* at 65° ended with the complete decomposition of the pigment.

Activated charcoal adsorbs phoenicine from the medium made slightly alkaline, but the product, recovered by solvent extraction, was completely unsatisfactory. The acidified substrate was therefore extracted with $CHCl_3$, which solution was then re-extracted with dilute aqueous $NaHCO_3$. This process was repeated several times [cf. Friedheim, 1933].

The final $CHCl_3$ solution, dried over anhydrous Na_2SO_4, was evaporated *in vacuo* and the phoenicine recovered as microscopic yellow prisms. This, after a few further recrystallizations from pure benzene, yielded a mixture of prisms and prismatic needles, which when heated rapidly melted at 231–232° (decomp.), first becoming dark in colour and finally black. (Found: C, 61·72, 61·24; H, 3·68, 3·72 %; mol. wt. 267, 271. $C_{14}H_{10}O_6$ requires C, 61·31; H, 3·65 %; mol. wt. 274.)

For purposes of comparison of the metabolism of the organism on the three media, the dry mycelium and pigment yields, calculated in g. per litre of initial substrate are shown in Table 2.

Table 2

Medium	Mycelium g.	Pigment g.
Raulin-Thom	17·1	0·082
Czapek-Dox	13·2	0·510
Raulin-Dierckx	10·8	0·261

A further batch of bottles was inoculated and allowed to grow over a period of 7 weeks. The final *p*H of the medium (Czapek-Dox) was 8·1 and the mycelium and phoenicine yields per litre were 13·4 and 0·204 g. respectively. From this, it appears that, lacking other sources of carbon, *P. phoeniceum* will utilize phoenicine.

The concentration of $NaNO_3$ in the Czapek-Dox medium was found to have a direct influence on the production of mycelium and pigment. Accordingly, individual batches of 2 l. of the solution were made up, containing concentrations of $NaNO_3$ ranging from zero to 4 g./l. by 0·5 g. increments. Sterilized and inoculated as before, these were allowed to grow for 18 days and then treated as already described. The figures obtained are incorporated in Table 3.

Table 3

Conc. of $NaNO_3$ g./l.	Mycelium g./l.	Phoenicine g./l.
0·0	No growth	—
0·5	5·1	0·174
1·0	8·9	0·345
1·5	11·5	0·540
2·0	12·8	0·504
2·5	12·2	0·320
3·0	10·4	0·202
3·5	10·2	0·148
4·0	9·5	0·057

Therefore, while pigmentation is practically entirely inhibited on a Czapek-Dox medium, containing 4·0 g. $NaNO_3$/l., the optimum yield is obtained on a solution, containing 1·5 g./l.

2. *PENICILLIUM RUBRUM* GRASBERGER-STOLL

Penicillium rubrum, included in Thom's classification of the "Biverticillata symmetrica", produces, on acid Czapek-Dox synthetic medium, two pigments [Posternak, 1939], the first, yellow, diffusible and only slightly affected by changes in *p*H and, in older cultures, a compound which proves to be phoenicine. The organism was therefore grown on the three synthetic media, acid Czapek-Dox, Raulin-Thom and neutral Raulin-Dierckx, for purposes of comparing the general metabolism and on Czapek-Dox solution containing varying concentrations of $NaNO_3$ and $MgSO_4$, $7H_2O$ for the influence of nitrogen and magnesium concentrations (cf. Part I).

Experimental

The culture of *P. rubrum* was obtained from the Centraalbureau voor Schimmelcultures. The yellow mycelium of the 2nd day, on malt agar, gave way to green conidia on the 3rd while pigmentation was apparent in 3–4 days. Later a plentiful separation of loose spores commences.

Nine litres of each of the three media were made up as described in Part I and distributed in 100 ml. amounts in 20 oz. flat bottles, which were plugged with cottonwool, sterilized by steaming on each of three consecutive days and inoculated with spore suspension of *P. rubrum*, grown for 4–5 days on malt-agar. The bottles, incubated in the dark at 20°, were allowed to grow for 18 days.

The following details were noted during growth: On the Czapek-Dox medium, mycelium formation became apparent in 2 days with a white overgrowth of the liquid in 3 days. The smooth light green felt of the 4th, changed to a heavily sporing darker green on the 5th day, there being no further change for the period of growth. The reverse, first yellow, altered to light brown and then gradually darkened in shade. The medium, first yellow, was faintly red on the 4th day and intense red on the 9th, there being no further change.

On the Raulin-Thom medium, the mycelium was slightly corrugated and yellow in colour on the 3rd day, changing to greenish yellow on the 4th and deepening to grey-green with blue-grey patches on the 12th. The reverse, first yellow, was brown on the 9th with gradual darkening in shade. Yellow in 4 days, the substrate altered through orange to orange-red on the 6th and to deep red on the 8th day.

The mycelial felt on the Raulin-Dierckx medium was corrugated and yellow on the 4th day and yellow-green on the 5th, deepening on continued growth. The yellow reverse changed slowly to a final brown. First faintly yellow, the medium deepened to golden yellow, without further change.

*p*H determinations were made at intervals of 3 days, the mixed contents of 4 bottles being used for one reading. In the case of the Czapek-Dox and Raulin-Thom media, the increase in acidity was too slow to obtain clearly defined values but, on the 18th day, the respective reactions were *p*H 2·9 and 2·6. Both these values are included in the *p*H range, 1·8–3·4, in which the change from yellow to red occurs in solutions of phoenicine (cf. Part I). The reaction of the Raulin-Dierckx solution altered from *p*H 7·0 to 1·9 in 6 days and showed an upward trend to the final reaction of *p*H 2·3.

After 18 days' growth, the mycelium was strained off, washed with a small quantity of water and, when pressed, dried *in vacuo*. In filtering, no precautions were taken to retain the loose spores. The acidified filtrate and washings were extracted with $CHCl_3$ and dilute $NaHCO_3$ solution (cf. Part I). The yellow diffusible pigment, mentioned by Posternak, is not present in quantity and, being less soluble in $NaHCO_3$ than is phoenicine, is partly retained by the $CHCl_3$ during extraction while the remainder separates out as a film on the walls of the vessel when the $NaHCO_3$ extracts are acidified. As the phoenicine, obtained on recrystallizing from benzene, melts sharply at 231–232°, it appears that this yellow pigment is completely lost in the extraction. The yields of phoenicine and dry mycelium for the three media are calculated per initial litre of substrate (Table 4).

Table 4

Medium	Mycelium g.	Phoenicine g.
Czapek-Dox	8·9	1·457
Raulin-Thom	12·4	1·370
Raulin-Dierckx	12·3	0·142

It was found that the age of the inoculating culture tended to diminish the yields; a 79-day old culture on malt agar when used for the inoculating spore suspension gave (Table 5):

Table 5

Medium	Mycelium g.	Phoenicine g.
Czapek-Dox	9·8	1·281
Raulin-Thom	11·5	1·060
Raulin-Dierckx	12·0	0·124

A further batch of 4 l. of Czapek-Dox medium was inoculated from a 6-day old culture and incubated as before for 24 days. Four bottles were taken at intervals of 3 days and the contents extracted, the yields of phoenicine being given in Table 6:

Table 6

Days of incubation	Phoenicine g./l.	Days of incubation	Phoenicine g./l.
0	—	15	1·043
3	—	18	1·403
6	0·150	21	1·260
9	0·590	24	1·183
12	0·703		

From these results, it seems that, as for *P. phoeniceum* (cf. Part I), 18 days are the optimum period of growth for *P. rubrum*, and further that this organism will also utilize phoenicine as a source of carbon.

In dealing with the influence of nitrogen concentration, individual batches of 2 l. of Czapek-Dox solution, containing amounts of $NaNO_3$, ranging from zero to 4 g./l. by 0·5 g. increments, were inoculated from 7-day old cultures and incubated for 18 days. Table 7 gives the production of phoenicine and dry mycelium per litre.

Table 7

$NaNO_3$ g./l.	Mycelium g.	Phoenicine g.
0	—	—
0·5	2·8	0·400
1·0	4·9	0·645
1·5	6·1	0·851
2·0	7·5	1·327
2·5	10·8	1·307
3·0	11·3	1·890
3·5	11·8	0·772
4·0	11·7	0·633

Two litre amounts of Czapek-Dox medium, containing 0, 0·1, 0·2, 0·3, 0·4 and 0·5 g. $MgSO_4$, $7H_2O$/l. were inoculated from 6-day old cultures and grown, under the usual conditions. The resultant values for mycelium and pigment production are contained in Table 8:

Table 8

Conc. of $MgSO_4$, $7H_2O$ g./l.	Mycelium g./l.	Phoenicine g./l.
0	2·1	0·220
0·1	8·3	1·560
0·2	9·4	1·568
0·3	10·5	1·848
0·4	8·7	1·436
0·5	9·2	1·360

Analysis of Tables 7 and 8 shows that the optimum concentrations of $NaNO_3$ and $MgSO_4$, $7H_2O$ are 3·0 and 0·3 g./l. respectively. A solution of the following composition was then made up and inoculated in the usual manner: glucose, 50 g.; $NaNO_3$, 3·0 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·3 g.; K_2HPO_4, 1·0 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water to 1000 ml.

After 18 days, the dry mycelium and phoenicine yields per litre of initial substrate, obtained as already described were: mycelium, 11·4 g.; phoenicine, 2·0 g. This result is of particular interest as the new yield shows a mean 45 % increase over those already obtained.

Summary

P. phoeniceum produces phoenicine more readily on synthetic media than on beerwort and in the case of one medium, acid Czapek-Dox, yields the pigment in greater quantity, viz. 51 mg./100 ml. as compared with 44 mg. on a beerwort substrate [Friedheim, 1938].

On Czapek-Dox and Raulin-Thom media, *P. rubrum* produces nearly equal quantities of phoenicine, quantities which are $2\frac{1}{2}$–3 times greater than the optimum yield on Czapek-Dox medium of *P. phoeniceum*. The organism is similarly, but not identically, influenced by *p*H and nitrogen concentration and gives a greatly increased yield of the pigment, when grown on a specially modified medium.

The authors are very much indebted to Prof. A. Robertson of Liverpool University for the helpful interest he has taken in this work.

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